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T-Zellen im Kontext von Autoimmunität und malignen Neoplasien

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Referat

T-Zellen als Teil der adaptiven Immunität sind von fundamentaler Bedeutung für die Immunabwehr gegen Pathogene und den Kampf gegen Tumore. Im Falle des Auftretens von Autoimmunität können beispielhaft für die rheumatoide Arthritis (RA) T-Zellen autoreaktiv agieren. Sie greifen körpereigene Strukturen an und sind maßgeblich an der Pathogenese der Erkrankung beteiligt. Bei Vorliegen einer Krebserkrankung wird das Immunsystem, u.a. krebsspezifische T-Zellen, vom Tumor kontrolliert und damit eine adäquate Bekämpfung verhindert. In den hier dargestellten Forschungsarbeiten wurde die Steuerung von T-Zell-Antworten zur Aufrechterhaltung ihrer Homöostase, zum Kampf gegen das maligne Melanom bzw. Nierenzellkarzinom und zur reaktiv übersteigerten Aktivität bei RA untersucht. Es galt dabei, sowohl krankheitsspezifische als auch übergeordnete Grundprinzipien der T-Zell-Immunologie zu entschlüsseln. Hierfür wurden T-Zellen aus dem Blut von Patienten/Patientinnen, gesunden Probanden/Probandinnen und aus Lymphorganen von Mäusen mit experimenteller Arthritis verwendet. Des Weiteren wurden biologische Zielstrukturen der Patienten/Patientinnen wie Haut und Synovialgewebe analysiert und die gewonnenen experimentellen Ergebnisse mit den klinischen Daten der Patienten/Patientinnen korreliert. Analysen an Zelllinien verschiedener Gewebetypen vervollständigten die Untersuchungen. Wesentliche Erkenntnisse des ersten Schwerpunktes „T-zelluläre Beteiligung bei der RA“ ergaben sich aus der Beschreibung und Charakterisierung multifunktioneller doppelt positiver ($CD4^+CD8^+$) T-Zellen. Außerdem konnten *LIR-1* und das murine Ortholog *PIR-B* als wichtige T-Zell-Regulatoren bei der RA identifiziert werden. Eine Infektion mit dem Cytomegalievirus führte bei RA-Patienten/-Patientinnen einhergehend mit hochreaktiven T-Zellen zu einem schwereren Krankheitsverlauf. Im zweiten Schwerpunkt „T-zelluläre Beteiligung bei malignen Neoplasien“ wurde eine prominente Rolle von *B7-H4* für das Überleben von Melanompatienten/-patientinnen und die zytokingesteuerte Regulation von *B7-H1* im Nierenzellkarzinom identifiziert. Der Kostimulator *B7-H3* auf Keratinozyten ist an der T-Zell-Homöostase in der Haut beteiligt. Die Erkenntnisse der durchgeführten Forschungsarbeiten komplettieren das Verständnis der Beteiligung verschiedener T-Zell-Subpopulationen und Regulationsmechanismen von T-Zell-Antworten im Kontext von Autoimmunität und malignen Neoplasien. Neue Zielstrukturen für therapeutische Ansätze und für fortführende Forschungsarbeiten konnten mit der vorliegenden Habilitationsschrift aufgezeigt werden.

Bibliographische Beschreibung

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Abkürzungsverzeichnis (Abstände hinter die Abkürzungen)

ACPA	Anti-citrullinierte Peptid/Protein Antikörper
AMPK	Adenosinmonophosphat Kinase
APZ	Antigen-präsentierende Zellen
ATL	Akutes T-Zell-Lymphom
ATP	Adenosintriphosphat
B7-H-xy	B7 Familienmitglieder Ko-stimulatoren, -inhibitoren
BTLA	B und T-Lymphozyten Attenuator
CMV	Cytomegalievirus
CTLA-4	Zytotoxisches T-Lymphozyten assoziiertes Antigen 4
CX3CR1	Chemokinrezeptor 1, Fraktalkin-Rezeptor
DP T-Zellen	$CD4^+/CD8^+$ doppelt positive T-Zellen
DNA	Desoxyribonukleinsäuren
Fas/FasL	Todesrezeptorpaar, TNF Familie
HD	Gesunde Probanden
HLA	Humanes Leukozyten Antigen / humaner Haupthistokompatibilitätskomplex
HIV	Humanes Immundefizienz Virus
IFN	Interferon
IkB	Inhibitor von NFkB
IL-xy	Interleukin
ITIM	Immunrezeptor Tyrosinbasierendes Inhibitorisches Motiv
LFA-1	Lymphozyten Funktionsassoziiertes Antigen 1
LIR1	Leukozyten Immunoglobulin ähnlicher Rezeptor 1, auch CD85j, ILT2
MAPK	Mitogen aktivierte Proteinkinase
MCP-1	Monozyten chemotaktisches Protein-1
MHC	Major Histocompatibility Complex
MS	Multiple Sklerose
NFkB	Transkriptionsfaktor *Nukleusfaktor kappa B* leichte Kette Verstärker
NF-90	RNA bindendes Protein *Nukleusfaktor 90*
PD-1 (R)	Programmierter Zelltod-Rezeptor
PD-L1	Programmierter Zelltod-Ligand

PI3k	Phosphoinositid-3-Kinase
PIR-B	Gepaarter Immunoglobulin ähnlicher Rezeptor B
PKC	Proteinkinase C
PMA	Phorbolmyristin Azetat
RA	Rheumatoide Arthritis
RBP	RNA bindendes Protein
RNA	Ribonukleinsäure
Runx3	Runt verwandter Transkriptionsfaktor 3
SASP	Senszenz-assoziierter sekretorischer Phänotyp
SKG	Mausmodell mit Spontanmutation
SLE	Systemischer Lupus Erythematodes
SPF	Spezifisch pathogenfrei
STAT6	Signal Übermittler und Aktivator der Transkription 6
TAA	Tumor-assoziierte Antigene
TAM	Tumor-assoziierte Makrophage
TEMRA	Terminal differenzierte Effektor-Gedächtniszellen
TFh	T-Follikulare Helferzellen
TGF-β	Transformierender Wachstumsfaktor beta
Th	T-Helferzellen
Th1, 2, 9 , 17, 22	Numerisch differenzierte Subtypen der T-Helferzellen
ThPOK	Transkriptionsfaktor, T-Helfer induzierend, Zinkfinger beinhaltend
TIGIT	T-Zellen-Immunrezeptor mit Immunglobulin und ITIM Motiven
TLS	Tertiäre lymphoide Strukturen
TNF	Tumor-Nekrose Faktor
Treg	Regulatorische T-Zellen
TRAIL/ TRAILR	Tumor-Nekrose Faktor verwandte Apoptose induzierendes Rezeptorpaar
TZR	T-Zell-Rezeptor
αβ-, γδ TZR	Alpha-beta bzw. gamma-delta T-Zell-Rezeptor
wt	Wildtyp

1. Einleitung

1.1. T-Zellen als aktive Gestalter der Immunabwehr

T-Zellen, auch T-Lymphozyten genannt, bilden zusammen mit den B-Zellen die zelluläre Ebene des adaptiven Immunsystems. T-Zellen werden in 2 große Hauptgruppen, die $CD4^+$ und $CD8^+$ T-Zellen, eingeteilt. Des Weiteren werden Mikromilieu-assoziierte, funktionelle, lokal begrenzte und differenzierungsverankerte Subtypen beschrieben. Diese T-Zell-Subtypen unterscheiden sich phänotypisch durch Zelloberflächenmoleküle bzw. Differenzierungsmarker und durch ihre vielgestaltigen Funktionen. T-Zell-Subtypen repräsentieren keine starren, sondern plastische Phänotypen, d.h., sie können zu einem bestimmten Zeitpunkt an einem Entzündungsort dem einen oder anderen Subtyp angehören, wobei diese Zugehörigkeit in einem anderen immunologischen Kontext verändert werden kann. Dies setzt eine gewisse Langlebigkeit dieses Zelltyps voraus; und in der Tat sind T-Zellen der Gesamtlebenszeit verschiedener Organismen angepasst, wobei sie bis zu Jahrzehnten überleben und in dem erforderlichen Immungeschehen entsprechend aktiv werden können.

Die T-Zell-Entwicklung wird ab dem Stadium der Lymphozytenprogenitoren, die das Knochenmark verlassen haben, über verschiedene Stufen in unterschiedlich strukturierten Bereichen des Thymusgewebes vollzogen. Die unterschiedlichen T-Zell-Entwicklungsstufen sind durch eine differentielle Expression der Korezeptoren CD4 und CD8 auf der Zelloberfläche gekennzeichnet. Um T-Zellen zu entwickeln, die als naive CD4 bzw. CD8 einzel positive T-Zellen den Thymus verlassen, sind vor allem 2 Transkriptionsfaktoren verantwortlich. Dies sind die Transkriptionsfaktoren *ThPOK* für $CD4^+$ T-Zellen und *Runx3* für $CD8^+$ T-Zellen. *ThPOK* bindet das CD8a Verstärkerelement (engl. Enhancer) und unterdrückt die Transkription von CD8 und anderen zytotoxischen Effektorgenen. Parallel bindet es an das geneigene Dämpfer- (engl. Silencer) Element und steuert dadurch die kontinuierliche Eigentranskription, was letztlich zu einer CD4 T-Zellentwicklung führt (Egawa and Littman 2008, Wang et al. 2008, Mucida et al. 2013). Die Funktion von *Runx3* für die CD8 T-Zellentwicklung ist ähnlich.

Naive T-Zellen werden in die Peripherie entlassen und können dort nach einer Aktivierung in die verschiedenen Differenzierungsstadien (Effektor-Gedächtniszellen, zentrale Gedächtniszellen und terminal differenzierte Effektorzellen) übergehen.

(Abb.1). Die Fähigkeit der T-Zellen, infolge einer Aktivierung in verschiedene Differenzierungsstufen zu wechseln, diese zu erhalten bzw. kontextspezifisch anzupassen, begründet die enorme Leistung des adaptiven Immunsystems, den vielfältigen Aufgaben adäquat zu begegnen.

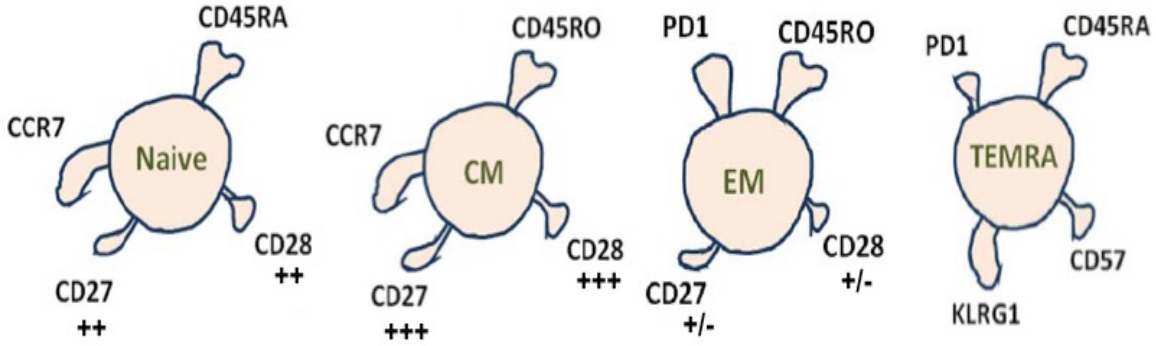

Abb.1 Darstellung der T-Zell-Differenzierungsmarker. Differenzierungsstufen CD4 und CD8 positiver T-Zellen werden durchflusszytometrisch entsprechend der Expression nachfolgend aufgeführter Oberflächenmarker unterschieden: naiv (naive): $CD45RA^{+}CCR7^{+}CD27^{++}CD28^{++}$, zentrale Gedächtniszellen (CM) $CD45RA^{-}CD45RO^{+}CCR7^{+}CD27^{+++}CD28^{+++}$, Effektor-Gedächtniszellen (EM) $CD45RA^{-}CD45RO^{+}CCR7^{-}CD27^{+/-}CD28^{+/-}PD1^{+}$, terminal differenzierte Effektor-Gedächtniszellen (TEMRA) $CD45RA^{+}CCR7^{-}CD27^{-}CD28^{-}PD1^{+}CD57^{+}KLRG1^{+}$, modifiziert nach (Larbi and Fulop 2014)

Im gealterten Organismus ist der Thymus als primäres lymphatisches Organ zurückgebildet. Naive T-Zellen in der Peripherie übernehmen eine Art Stammzellfunktion und erhalten das T-Zell-Kompartiment durch homöostatische Proliferation, periphere Selektion und Aktivierung verschiedener Differenzierungsprogramme (Yanes et al. 2017).

T-Zellen werden spezifisch über im HLA-Kontext präsentierte Peptide (auch Antigene oder Peptidantigene), die nach Spaltung aus Proteinen entstehen, über ihren T-Zell-Rezeptorkomplex (*TZR*) aktiviert. Eine T-Zelle trägt jeweils nur einen bestimmten T-Zell-Rezeptor, der durch Kreuzreaktivitäten allerdings spezifisch für mehrere Peptide sein kann (Birnbaum et al. 2014). Der größte Teil der T-Zellen des Blutes trägt TZR mit *αβ*-Ketten, nur 1-5 % der T-Zellen des Blutes exprimieren TZR mit *γδ* Ketten. T-Zellen des *γδ* Ketten-Typs zeichnen sich durch ihre Fähigkeit aus, Nicht-Peptidantigene, wie z.B. Phosphoantigene des Isoprenoid-Metabolismus HLA-Antigen-Komplex-unabhängig zu erkennen. T-Zellen mit *γδ TZR* sind vorrangig in mukösen Geweben, z.B. der Darmmukosa oder im Epithel der Lunge lokalisiert.

Die Peptidpräsentation für die Aktivierung von T-Zellen des αβ-Ketten-Typs wird von professionellen Antigen-präsentierenden Zellen (APZ) (Abb. 2, dendritische Zellen,

Monozyten/Makrophagen, B-Zellen) oder aber von nicht-professionellen Antigen-präsentierenden Zellen (konstitutiv HLA Klasse I tragende Körperzellen verschiedener Organe, Körperzellen, die durch Zytokine HLA Klasse II exprimieren oder *γδ* T-Zellen, über deren HLA Klasse I Expression) übernommen. Peptidantigene werden für die Präsentation auf der Zelloberfläche auf Moleküle des Haupthistokompatibilitätskomplex (*HLA*) geladen, nachdem eine komplexe intrazelluläre Antigen-Prozessierung mit Passage des Ubiquitin-Proteasoms und des lysosomalen Systems erfolgt ist (Wieczorek et al. 2017).

Im Allgemeinen werden CD4 positive T-Zellen (T-Helferzellen) durch einen Antigen-HLA-Komplex der *HLA-Klasse II* Moleküle, die bevorzugt extrazelluläre/exogene Peptide präsentieren, aktiviert. CD8 positive, sogenannte zytotoxische, T-Zellen hingegen werden durch einen Antigen-HLA-Komplex unter Beteiligung der *HLA-Klasse I* Moleküle, die bevorzugt zytosolische/endogene Peptide präsentieren, aktiviert.

Die Funktion der Korezeptoren CD4 und CD8 liegt in der Stabilisierung des Peptid-*HLA-TZR* Komplexes auf der Zelloberfläche und in der Assoziation verschiedener intrazellulärer Signalmoleküle, die zur Aktivierung von T-Zellen führen (Doyle and Strominger 1987, Barber et al. 1989, Salter et al. 1990). Eine komplexe Kaskade verschiedener Signalmoleküle führt letztlich zur klonalen Expansion und zu verschiedenen T-zellulären Effektorfunktionen, wie Zytokinsekretion, B-Zellaktivierung oder die Apoptoseinduktion in Zielzellen.

T-Zellen finden sich im Körper nicht nur im Blut, in primären (Knochenmark, Thymus) und sekundären Lymphorganen (Milz, Lymphknoten, Tonsillen, Peyerschen Plaques, Appendix), sondern in der Haut, dem Intestinaltrakt und der Lunge. Die Verteilung der unterschiedlichen T-Zell-Differenzierungsstadien ist in den Geweben stark unterschiedlich (Sathaliyawala et al. 2013).

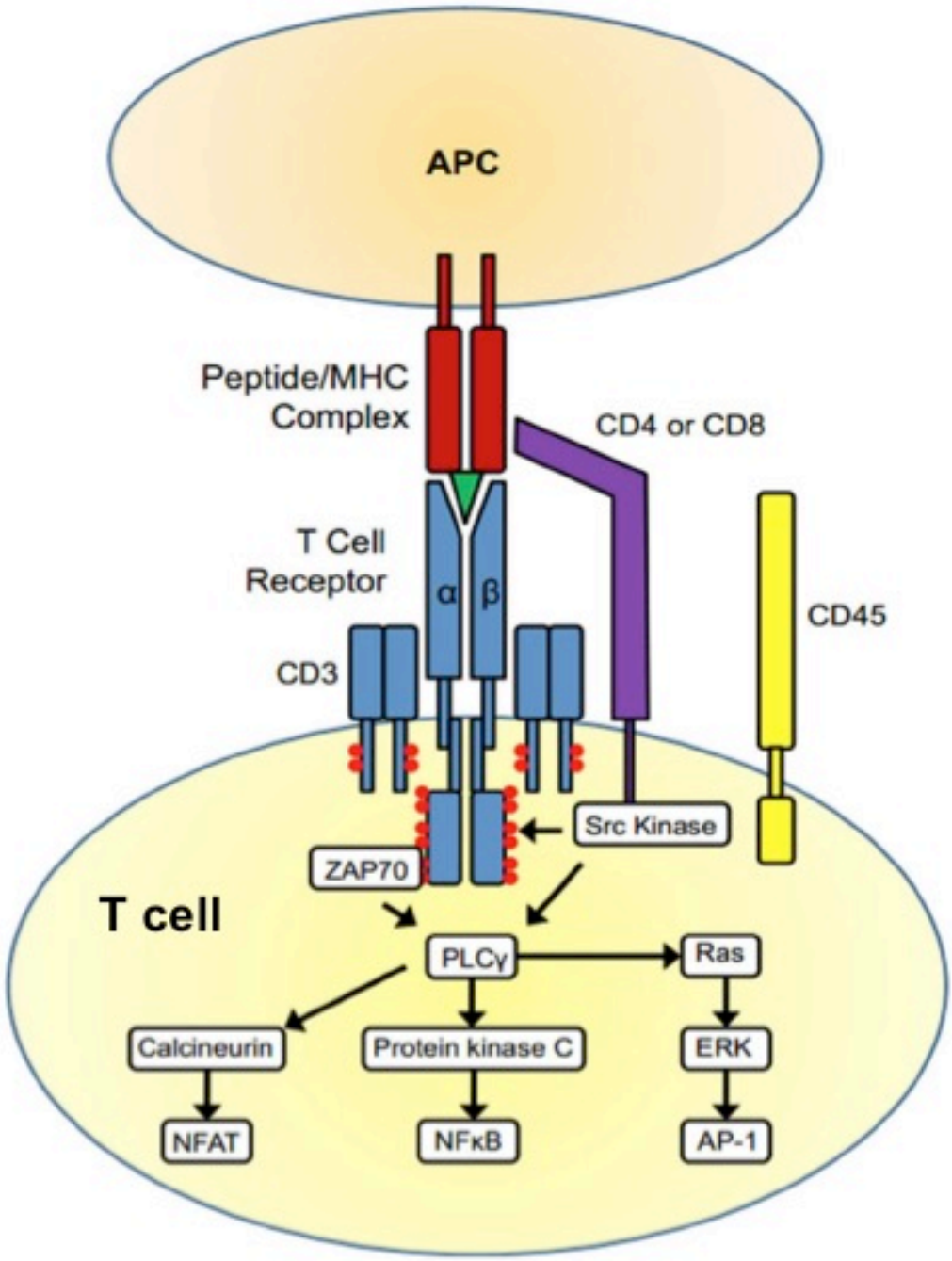

Abb.2 Schema der T-Zell-Aktivierung. Die Ketten des T-Zell-Rezeptorheterodimers und dessen Ko-Rezeptoren CD4 bzw. CD8 sind assoziiert mit Tyrosinkinasen. Zahlreiche Signalmoleküle (u.a. Src Kinasen und ZAP70) sind an der Weiterleitung des durch die Bindung des TZRs an Peptid-HLA Komplexe initiierten Aktivierungssignals, beteiligt. Sie führen letztlich zu multiplen zellulären Veränderungen, die für die T-Zell-Effektorfunktionen notwendig sind. Peptide/MHC-Complex = Peptide/HLA-Komplex (Whitacre et al. 2012).

1.1.1.CD4$^+$ T-Helferzellen

CD4 positive T-Helferzellen (*Th*) haben vielgestaltige Aufgaben im adaptiven Immunsystem, die durch eine Aufgabenteilung spezialisierter Th-Untergruppen möglich wird. *Th*-Zellen sezernieren eine Vielzahl verschiedener Zytokine. Z.B. leisten sie den B-Zellen Hilfe (durch *Th2*-Zytokine) bei der Antikörperproduktion, aktivieren u.a. Makrophagen (durch *Th1*-Zytokine) vor allem im Zuge der Erkennung, Prozessierung und Präsentation von exogenen Antigenen bakteriellen Ursprungs. *Th17*-Zellen bilden eine Spezialeinheit, die vorrangig für die Bekämpfung von Pilzen, aber auch Bakterien in der Haut und auf mukösen Oberflächen verantwortlich sind (Wacleche et al. 2017). T-Follikulare Helferzellen (*TFh)* wurden anhand ihres Transkriptionsprofils und ihrer Fähigkeit die B-Zellen zu unterstützen, bei fehlendem Vorkommen der *Th2* T-Zellen, als eigene T-Helfer-Zellpopulation mit Lokalisation im Follikel und Blut identifiziert (Ma and Phan 2017). CD4$^+$ *Treg* Zellen sind zusätzlich zu anderen Funktionen für die Aufrechterhaltung der peripheren und zentralen Toleranz als Mechanismus zur Verhinderung der Autoimmunität zuständig (Kraj and Ignatowicz 2017). Weitere CD4 *Th*-Subzelltypen, die sich durch die Produktion von *IL-9* (*Th9*) bzw. *IL-22* (*Th22*) auszeichnen, wurden in jüngerer Vergangenheit identifiziert (Geginat et al. 2014).

Nach neueren Erkenntnissen ist davon auszugehen, dass eine Differenzierung naiver CD4$^+$ T-Zellen zu *Th1, Th2, Th17, TFh* und *Treg*-Zellen in bestimmten Zwischenstufen einer Reprogrammierbarkeit unterliegt, die durch das vorhandene Zytokinmilieu (maßgeblich *IL-2, IL-4, IL-6, IL-12, IL-21*, *IFNγ* und *TGF-β*) gesteuert wird (DuPage and Bluestone 2016). Die Zytokin-mediierte Plastizität der T-Helferzellpopulationen sorgt für die notwendige Flexibilität der adaptiven T-Zell-Immunität.

Im Falle von Immunpathologien wurden *Th1/Th17*-T-Zellen vor allem bei Autoimmunerkrankungen wie Multipler Sklerose (MS), Diabetes mellitus Typ 1 oder auch rheumatoider Arthritis (RA) als Effektor-T-Zellen identifiziert (Crane and Forrester 2005). *Th2* Zytokine produzierende T-Zellen kennzeichnen die Autoimmunkrankheit Systemischer Lupus Erythematodes (SLE) und weitere Immunpathologien wie Allergien, Asthma und reaktive Arthritis (Quandt 2006). *TFh*

T-Zellen zeigen Funktionsstörungen bei diversen Autoimmunkrankheiten, wie z.B. der RA, SLE und der MS (Ma and Deenick 2014).

1.1.2. CD8$^+$ Zytotoxische T-Zellen

Ihrem Namen entsprechend besteht die Hauptaufgabe von CD8$^+$ T-Zellen darin, Zielzellen zu töten. Dieser Vorgang wird nach Aktivierung durch den entsprechenden TZR-HLA-Antigenkomplex und unter Einschaltung verschiedener molekularer Signalwege (über Perforin/Granzym bzw. *TRAIL-R/TRAIL* oder *Fas/FasL*) gezielt und gerichtet eingeleitet (Martinez-Lostao et al. 2015). Zu tötende Zellen sind meist viral infiziert oder hinsichtlich ihrer Expression von Tumorantigenen verändert. Darüber hinaus können CD8$^+$ T-Zellen suppressorische Aktivitäten entwickeln, wobei sie dendritische Zellen in einen tolerogenen Zustand versetzen. Durch diesen Prozess werden CD4$^+$ T-Zell-Antworten blockiert (Xu et al. 2016).

Die Entscheidung, ob eine naive CD8 T-Zelle zu einer kurzlebigen Effektorzelle oder zu einer langlebigen Gedächtniszelle differenziert, wird durch viele Faktoren, wie die Stärke des TZR Signals, beteiligte kostimulatorische Moleküle, Transkriptions-faktoren und das sogenannte immunologische Mikromilieu bedingt (Chang et al. 2014). Aus einer naiven T-Zelle mit einer *TZR*-Spezifität somit sowohl Effektor-T-Zellen als auch Gedächtnis-T-Zellen (Gerlach et al. 2010). Verschiedene Modelle beschreiben die Wege dieser Differenzierung, wobei im wesentlichen 2 dieser Modelle im Fokus stehen. Dies ist zum einen das Modell der Abspaltung von Gedächtniszellen aus naiven T-Zellen direkt nach der T-Zell-Aktivierung und zum anderen das Modell der Generierung von Gedächtniszellen aus vorherigen Effektorzellen. Changs Ergebnisse einer asymmetrischen ersten Zellteilung unterstützen das erste Modell (Chang et al. 2007). Neuste Forschungsarbeiten an humanen Gelbfieber-spezifischen T-Zellen hingegen belegen, dass Gedächtniszellen aus stark proliferierenden Effektorzellen differenzieren, die Effektormoleküle abschalten und Transkriptionsprofile naiver Zellen neu erwerben (Akondy et al. 2017). Ebenfalls von höchster Wichtigkeit sind in diesem Kontext Erkenntnisse zu CD8 T-Zellen mit einem Stammzell-ähnlichen Potential, die als distinkte T-Zell-Population neben konventionellen Gedächtniszellen nach einer Infektion zurückbleiben. Diesen T-Zellen wird ähnlich wie von Chang 2007 postuliert nach Aktivierung eine sehr frühe Abspaltung von den Effektor-T-Zellen attestiert (Restifo 2014).

Jüngste Forschungsarbeiten zeigen eine beachtenswerte Abhängigkeit der CD8 T-Zell-Differenzierung von der Aktivität des Proteasoms. Das Proteasom ist ein Multiproteinkomplex, der für den geordneten Abbau von Proteinen in der Zelle und damit fundamental für das Zellüberleben verantwortlich ist (Finley 2009). Effektor $CD8^+$ T-Zellen weisen eine geringe Proteasomaktivität auf, wohingegen Gedächtnis $CD8^+$ T-Zellen im präklinischen Mausmodell eine hohe Proteasomaktivität besitzen (Widjaja et al. 2017). Manipulatoren der Proteasomaktivität, denen derzeit ein hohes therapeutisches Potential zugesprochen wird (Cromm and Crews 2017), könnten daher maßgeblich in die T-Zell-Differenzierung eingreifen.

Die Identifikation von $CD4^+$ T-Zellen, die nicht wie zuvor beschrieben Helferfunktionen übernehmen, sondern zytotoxisch wirken, und $CD8^+$ T-Zellen, die nicht töten, aber B-Zellhilfe leisten, zeigt eine höhere Komplexität T-Zell-vermittelter Immunantworten auf (Wagner et al. 1998, Brown 2010). Diese T-Zellen können somit auf ein vorhandenes Antigenspektrum sowohl im Kontext einer *HLA* Klasse I- ($CD8^+$) als auch einer *HLA* Klasse II ($CD4^+$) vermittelten Antigenpräsentation mit der auf die Situation angepassten Effektorreaktion immunologisch tätig werden.

1.1.3. $CD4^+CD8^+$ doppelt positive (DP) T-Zellen

$CD4^+CD8^+$ DP T-Zellen gehören als intermediäre Entwicklungsstufe der T-Zell-Reifung zum typischen Bild einer durchflusszytometrischen T-Zell-Analyse im Thymus (Issuree et al. 2017).

Periphere DP ($CD4^+CD8^+$) T-Zellen sind mit einer Häufigkeit von 0,5-1% der T-Zellen im Blut von gesunden Probanden zu finden (Parel and Chizzolini 2004). Bereits vor über 25 Jahren wurden sie erstmalig beschrieben (Paliard et al. 1988).

Die Zahl, die Antigenspezifität, das Effektorpotential und die Lokalisation dieser DP T-Zellen unterscheiden sich vom gesunden Normalbild im Falle von Autoimmunität, Virusinfektionen und Tumorerkrankungen, wodurch eine pathologische Rolle dieser Zellen impliziert wird (Nascimbeni et al. 2004, Parel et al. 2007, Desfrancois et al. 2010).

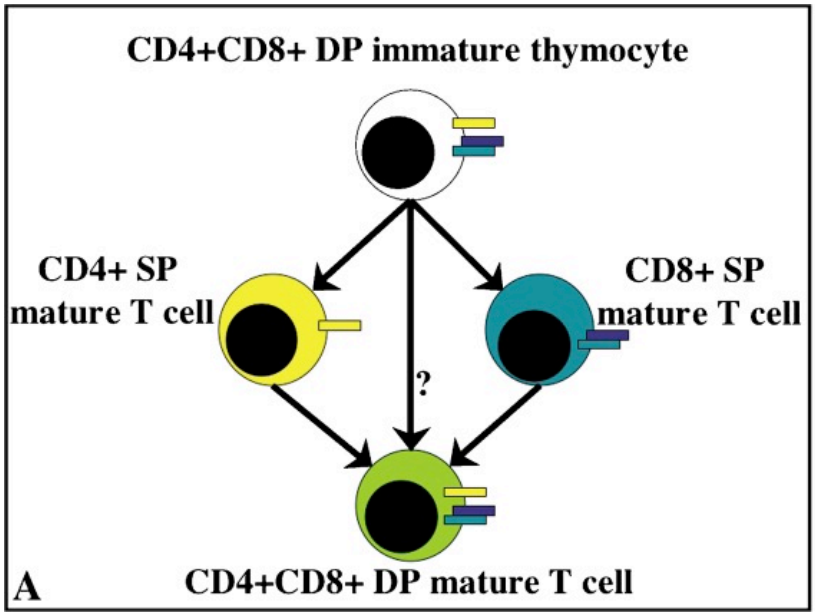

Abb.3 Darstellung der DP ($CD4^+CD8^+$) T-Zellen Doppelt positive T-Zellen während der T-Zell-Entwicklung im Thymus (immature Thymozyten) und als gereifte (mature) T-Zellen, die aus einzel $CD4^+$ bzw. $CD8^+$ T-Zellen hervorgehen (Parel and Chizzolini 2004)

Obwohl noch nicht abschließend verstanden, wird davon ausgegangen, dass diese T-Zellen nicht dem Stadium unreifer $CD4^+CD8^+$-Thymozyten entspringen, sondern bereits aus einfach-positiven $CD4^+$ bzw. $CD8^+$ T-Zellen in der Peripherie entstehen (Abb.3).

Diese Hypothese wird durch das Fehlen von Markern unreifer, sich noch entwickelnder T-Zellen (Nascimbeni et al. 2004), und durch eine ähnlich hohe *TZR*-Dichte auf $CD4^+CD8^+$-T-Zellen wie auf $CD4^+$ bzw. $CD8^+$ einzel positiven peripheren T-Zellen (Paliard et al. 1988) unterstützt. Hinweise für das Entstehen DP (s.o.) T-Zellen in der Peripherie bietet ebenfalls eine Arbeit über Patienten/Patientinnen, die unter einem akuten T-Zell-Lymphom leiden. In ihnen exprimieren die entarteten peripheren $CD4^+$ T-Zellen im Verlauf der Erkrankung zusätzlich das CD8 Molekül, ohne unmittelbar zuvor dem Thymus entsprungen zu sein (Yamada et al. 1984).

Auf Transkriptionsfaktorebene konnte gezeigt werden, dass die für den Thymus zunächst als exklusiv beschriebene Ko-Expression von *ThPOK* oder *Runx3* auch für periphere T-Zellen zutreffen kann. Eine gleichzeitige Expression beider Marker ist folglich möglich, womit eine weitere Erklärung für das Zustandekommen peripherer $CD4^+CD8^+$ T-Zellen geliefert wurde (Reis et al. 2013).

DP T-Zellen (Colombatti et al. 1998, Ghia et al. 2007) steigen ähnlich anderen T-Zell-Populationen zahlenmäßig mit dem Alter an.

Es werden 3 Subgruppen $CD4^+CD8^+$ DP T-Zellen, die sich durch eine unterschiedliche Korezeptorstärke auszeichnen ($CD4^{++}CD8^+$, $CD4^{++}CD8^{++}$ und $CD4^+CD8^{++}$), differenziert (Parel and Chizzolini 2004).

Im Gegensatz zu anderen altersassoziierten T-Zell-Populationen werden DP T-Zellen als multifunktionell charakterisiert. Es konnte beispielsweise gezeigt werden, dass intratumorale DP T-Zellen in Melanompatienten/-patientinnen eine hohe Produktion an pro-inflammatorischem *TNF*α und anti-inflammatorischen *IL-4, IL-5* und *IL-13* aufweisen und DP T-Zellen autologe Melanomzellen töten können (Desfrancois et al. 2010). In HIV Patienten/Patientinnen weisen DP T-Zellen eine Multifunktionalität auf, die unabhängig von Immunantworten anderer T-Zell-Populationen ist (Frahm et al. 2012). Sehr interessant in diesem Zusammenhang ist außerdem eine Arbeit aus dem Jahr 2007 an Patienten/Patientinnen mit systemischer Sklerose, in der gezeigt wurde, dass DP T-Zellen sowohl *IFN*γ als auch *IL-4* produzieren und zytotoxische parallel zu B-Zellhelferaktivität aufweisen (Parel et al. 2007). Die Tatsache, dass DP T-Zellen durch die Expression von CD4 und CD8 Molekülen mit Antigenen, die auf *HLA-I* und *HLA-II* exprimiert werden, interagieren können, ermöglicht ihnen ein weitaus breiteres Wirkspektrum als einzel $CD4^+$ bzw. $CD8^+$ positiven T-Zellen.

1.2. Die Steuerung von T-Zell-Antworten

T-Zell-Antworten müssen für eine optimale protektive Immunität bei paralleler Verhinderung von Autoimmunität sorgfältig gesteuert werden. Diese Steuerung wird maßgeblich durch Liganden und Rezeptoren der Kostimulatoren, z.B. der *B7*-Familie bewerkstelligt, welche kostimulatorische bzw koinhibitorische Funktionen warnehmen (Schildberg et al. 2016). Gemeinhin versteht man unter kostimulatorischer Wirkweise die Unterstützung der T-Zell-Antworten, wobei koinhibitorische Signale die T-Zell-Antwort trotz spezifischer Antigenerkennung über den *TZR*-Peptid-*HLA* Komplex unterdrücken (Abb.3). T-Zell-Aktivierung ist daher abhängig von der Antigenspezifität und der korrekten Präsentation des Antigens über den *TZR*-Peptid-*HLA* Komplex (Signal 1), aber auch von weiteren Signalgebern, wie den Molekülen der Kostimulatoren, die über Rezeptor-Ligand-Interaktionen (Signal 2) agieren (Abb.4). Die Liganden der Mitglieder der B7-Familie (*B7-H1=PD-L1*, *B7-H2*, *B7-DC*, *B7-H3*, *B7-H4*, *B7-H6*, *B7-H7*) werden zumeist von Antigen-präsentierenden Zellen exprimiert. Aber auch aktivierte T-Zellen (Khanna et al. 2016) bzw. Körperzellen (z.B.

Epithelzellen) (Cao et al. 2011) und Stroma-Zellen des Knochenmarks (Xu et al. 2011) können diese Moleküle exprimieren und zeigen damit eine weitere Ebene der Regulation von T-Zell-Antworten auf. Zytokine werden als Signal 3 der T-Zell-Aktivierung betrachtet. Ohne eine koordinierte Zytokinsekretion werden T-Zellen auch bei optimalen Signal 1 und 2 Impulsen nicht adäquat aktiviert (Curtsinger et al. 1999). Bemerkenswerterweise kann ein überschießendes Vorkommen an Zytokinen vor der „Abfrage“ von Signal 1 und 2 die Antigen-spezifische Aktivierung von $CD4^+$ T-Zellen verhindern (Sckisel et al. 2015).
Die Ligandenexpression kostimuatorischer/koninhibtorischer Moleküle, beispielhaft für *PD-L1,* ist vielschichtig, geschieht über Zytokine, vor allem über Typ II Interferone wie das *IFNγ* (Blank et al. 2005) und über transkriptionelle und posttranskriptionelle Steuerung (Chen et al. 2016).

Adhäsionsmoleküle wie *LFA-1* sind zusätzlich wichtig für eine koordinierte T-Zell-Antwort und damit wichtige Steuerelemente der T-Zell-Aktivierung (Grakoui et al. 1999). Adhäsionsmoleküle unterstützen die Bildung der immunologischen Synapse. Die Formierung einer immunologischen Synapse, einer strukturellen Einheit an der Kontaktzone zwischen T-Zelle und APZ (Mukherjee et al. 2017), in der sich alle Moleküle der T-Zell-Aktivierung eng versammeln, wurde erstmals vor mehr als 20 Jahren beschrieben (Paul and Seder 1994).

Ein zukunftsweisender Forschungszweig, der T-Zell-Antworten steuert, ist die Möglichkeit der T-Zell-Aktivierung über Antigen-HLA-Komplexe, die in von dendritischen Zellen abgeschnürten Exosomen eingebettet sind (Robbins and Morelli 2014). Exosomen bilden 30-120 nm große extrazelluläre Vesikel, die von diversen Zellen abgeschnürt werden. Sie entfalten lokale und systemische Wirkung, indem sie Proteine, Peptide, *HLA*, *RNA*, *DNA* und Metabolite tragen bzw. einschließen, die in verschiedene Regionen des Körpers transportiert werden (Thery et al. 2009). Exosomen mit Antigen-*HLA*-Komplexen können T-Zellen entweder direkt (ohne weitere Zellhilfe, Kostimulation auf Exosomen vorhanden) oder indirekt (Hilfe von dendritischen Zellen, die als sogenannte *„Zuschauer"* engl. Bystander, Kostimulation liefern) aktivieren (Robbins and Morelli 2014).

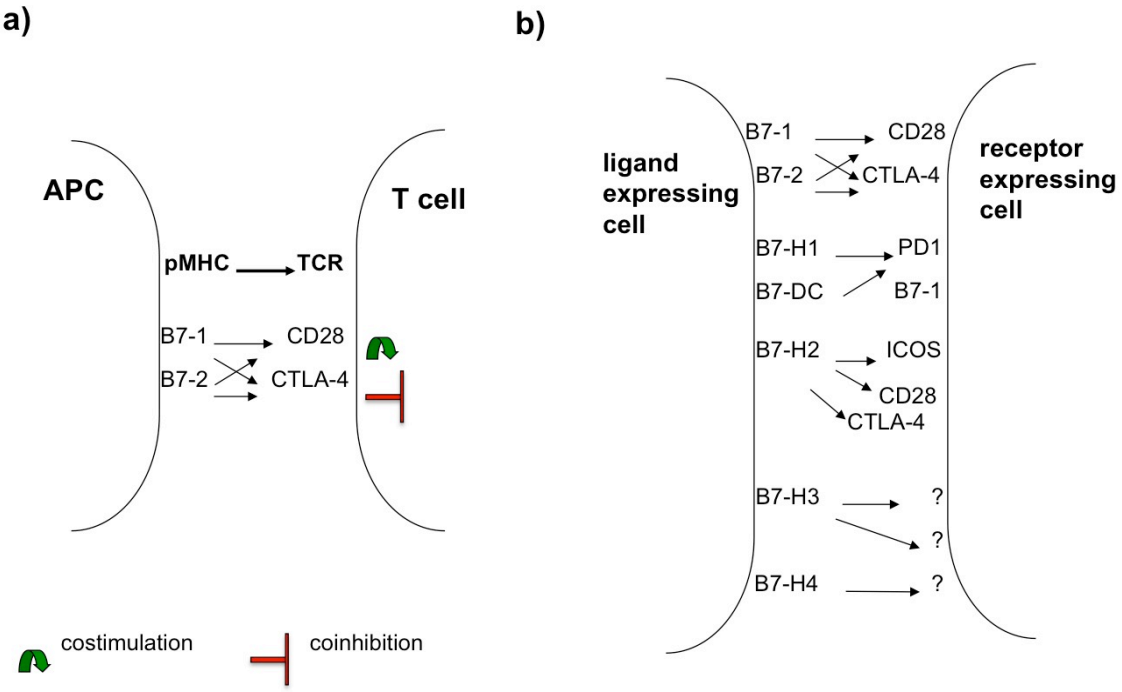

Abb.4 Signal 1 und Signal 2 der T-Zell-Aktivierung Die Aktivierung von T-Zellen erfolgt über a) Signal 1 = Peptide-MHC-TZR und Signal 2 = Kostimulation b) Ligand-Rezeptorbeziehungen von B7 Familienmitgliedern (Seliger and Quandt 2012)

Die Steuerung von T-Zell-Antworten findet über die geschilderten Prozesse hinaus auf mehreren weiteren Regulationsebenen statt, beispielsweise über

posttranslationale Modifikationen bestimmter Effektorgene (Perforin, Granzym, *IFNγ*, *IL-2*) durch RNA-bindende Proteine (*RBP*), non-coding *RNAs* und cis-regulatorische Elemente (Salerno and Wolkers 2015). Interessanterweise konnte gezeigt werden, dass es nach Kreuzvernetzung von *CD28* bzw. *LFA-1* durch *NF-90*, ein *RBP*, zur mRNA Stabilisierung von *IFNγ* und *IL-2* kommt (Shim et al. 2002, Wang et al. 2006).
T-Zell-Abschaltung, respektive Stopp von *IFNγ* Produktion, wird ebenfalls posttranslational durch Bindung eines *mRNA* abbauenden Moleküls Tristetrapolin (TTP) gesteuert (Ogilvie et al. 2009).
Neueste Forschungsarbeiten dokumentieren die Anpassung des T-Zell-Metabolismus über *AMPK,* ein Regulator intrazellulärer *ATP* Level*,* als Steuerelement von T-Zell-Antworten (Ma et al. 2017).

Darüber hinaus haben Entwicklungen unserer modernen Gesellschaft, wie ein verstärktes Auftreten von Adipositas (Green and Beck 2017) und das längere Überleben (Yanes et al. 2017), einen profunden Einfluss auf das T-zellimmunologische Aktionspotenzial durch Veränderungen in der Homöostase und Plastizität.

1.3. T-Zell-Immunität im Melanom und Nierenzellkarzinom

Melanome und Nierenzellkarzinome sind solide, immunogene Tumore, die komplexe Interaktionen mit dem Immunsystem eingehen.

T-Zellen erkennen tumorassoziierte Antigene (TAA) auf Antigen-HLA Komplexen, die sich aus Proteinen verschiedener biologischer Klassen rekrutieren. Es werden Differenzierungsantigene, gewebsspezifische Antigene, mutierte Antigene die im Zusammenhang mit der Onkogenese bzw. ursächlich für das Tumorwachstum verantwortlich sind, unterschieden (Pitcovski et al. 2017). Im Tumormikromilieu finden sich bei immunogenen Tumoren zahlreiche Vertreter des Immunsystems, unter ihnen $CD4^+$ und $CD8^+$ T-Zellen. Entscheidend für den Verlauf der Erkrankung und die Therapierbarkeit der Tumore sind dabei nicht allein die Anzahl der Immunzellen, sondern das Verhältnis verschiedener Populationen zueinander und die genaue Lokalisation im Tumorgewebe (Bindea et al. 2013). T-Zellen werden häufig in lokalen angrenzenden Lymphknoten aktiviert und wandern dann ins Tumorgewebe ein. Es kann aber ebenfalls im Tumor direkt zu einer Antigen-spezifischen Primäraktivierung oder einer „Zuschauer“ gleichbedeutend mit einer Antigen-unspezifischen T-Zell-Aktivierung kommen.

Die Tumore können Zellen der angeborenen und erworbenen Immunität für ihre Entwicklung nutzen. T-Zellen und andere Immunzellen, die sich gegen den Tumor richten, können in ihrer Funktion blockiert werden, wodurch Tumore sich der Bekämpfung durch das Immunsystem entziehen (Chen and Mellman 2017).

Tumorzellen, u.a. Melanome und Nierenzellkarzinome, exprimieren Kostimulatoren/Koinhibitoren, beispielsweise der B7-Familie, wodurch sie direkt T-Zell-Antworten steuern können (Seliger and Quandt 2012). Die Expression von *PD-L1* wird durch verschiedene Signalwege, wie den *PI3k* und *MAPK* Weg, über Zytokine und epigenetisch beispielsweise über microRNAs kontrolliert (Atefi et al. 2014, Chen et al. 2016). Chemotherapeutika und Radiotherapien können wissenswerterweise ebenfalls die Expression und damit Funktion von kostimulatorischen Molekülen, wie für *PD-L1* gezeigt, modulieren (Deng et al. 2014, Funaki et al. 2017).

Ein Zusammenhang zwischen dem seneszenten Immunsystem und dem vermindert erfolgreichen Kampf des Immunsystems gegen Krebs wurde und wird zunehmend entschlüsselt (Hurez et al. 2017).

Moderne Krebstherapie adressiert das Immunsystem, im Besonderen die T-Zellen. Moleküle der Kostimulation stehen dabei im Mittelpunkt, es wird versucht koinhibitorische Moleküle wie *CTLA-4* und *PD-1* mittels spezifischer Antikörper (Ipilimumab, Nivolumab oder Pembroluzimab) zu blockieren, um eine Inhibition/Abschaltung spezifischer T-Zell-Antworten gegen den Tumor aufzuheben (Mazza et al. 2017, O'Reilly and Larkin 2017).
Diese neuen Immuntherapien führen zu guten Erfolgen bei der Krebsbekämpfung einiger Patienten/Patientinnen, führen jedoch bei anderen Patienten/Patientinnen zu Autoimmunphänomenen wie beispielsweise zur rheumatoiden Arthritis (Cappelli et al. 2017). Dies ist vermutlich der T-Zell-Antigen-unspezifischen Aufhebung wichtiger T-Zell-Kontrollelemente geschuldet.

1.4. Rheumatoide Arthritis – eine T-Zell-gesteuerte Autoimmunerkrankung

Die rheumatoide Arthritis ist eine systemische Autoimmunerkrankung, bei der durch entzündliche Prozesse im Gelenk das Knorpel- und Knochengewebe geschädigt wird (McInnes and Schett 2011). Die entzündlichen Prozesse im Gelenk führen letztlich häufig zum Knorpelabbau und zur Knochendestruktion. Die zelluläre Beteiligung erstreckt sich über das angeborene und erworbene Immunsystem. Des Weiteren sind gelenksresidente Zellen wie Synoviozyten und Knorpelzellen im synovialen Gewebe an der Pathogenese beteiligt (Angelotti et al. 2017).

In einem substanziellen Teil der RA-Patienten/-Patientinnen findet man Autoantikörper gegen posttranslational modifizierte Proteine (Nakken et al. 2017). Dazu zählen citrullinierte Proteine, die durch die Aktivität einer Peptidylarginin Deaminase, die Citrullin gegen Arginin tauscht, entstehen.

Die Bedeutung der T-Zellen für die RA wurde bereits vor über 30 Jahren entdeckt, als genetische Assoziationsstudien eine Prävalenz *HLA-DR1* (auch bekannt als shared epitope) und *-DR4* tragender Personen für die Entwicklung einer Arthritis aufdeckten (Stastny 1978, Gregersen et al. 1987). In jüngeren Tagen wird oft von multifaktoriellen Voraussetzungen für die Entwicklung einer RA gesprochen, die HLA-DR Moleküle gehören nach wie vor dazu (Messemaker et al. 2015).
Im Falle von Autoimmunerkrankungen richten sich aus noch ungeklärter Ursache $CD4^+$ aber auch $CD8^+$ T-Zellen gegen körpereigene Strukturen (Autoantigene), und lösen somit eine Kaskade an Ereignissen aus, die zur organspezifischen Zerstörung

führt. Neben den sehr intensiv studierten CD4 positiven T-Zellen, die Autoantigene erkennen, konnten in verschiedenen Autoimmunkrankheiten (RA, Diabetes mellitus, Multiple Sklerose) CD8 positive Zellen identifiziert werden, die aktiv in den pathologischen Prozess eingreifen (Wagner et al. 1998, Friese and Fugger 2009, Mallone et al. 2011). Für die Pathogenität CD4 positiver T-Helferzellen bei der RA ist das Verhältnis von *Th1*, *Th17* und *Treg* Zellen wichtig. Moderne immunologische Therapieverfahren bei der RA schließen anti-TNFα Antikörper (z.B. Adalimumab) und Antikörper gegen den *IL-6R* (z.B. Tocilizumab) ein (Smolen et al. 2017). Tocilizumab verhindert *IL-6* Rezeptorbindung, wodurch die Aktion des pleitropen Zytokins, welches u.a. *Th17* Differenzierung befördert und Treg-Zellen unterdrückt, inhibiert wird (Schinnerling et al. 2017).

1.5. Cytomegalovirusinfektionen (CMV) – T-zelluläre Veränderungen

Das Cytomegalievirus (CMV) gehört zur Familie der Herpesviridae und bleibt dem Menschen nach dem Erstkontakt als latente Infektion ein Leben lang erhalten. Die Latenzorte für den Virus sind zahlreich, es kann in Endothelzellen, renalen Epithelzellen, im Gewebe der Lunge und in Immunzellen myeloiden Ursprungs in einem ruhenden Zustand überleben (Gerna et al. 2004).

CMV Infektionen hinterlassen deutliche Spuren in T-zellulären aber auch in Natürlichen Killerzell-Kompartimenten des adaptiven/angeborenen Immunsystems in gesunden Probanden (Appay et al. 2008, Heath et al. 2016). Es kommt zu einem differenzierten T-zellulären Phänotyp mit erhöhtem Verlust des Kostimulators CD28 auf CD4 und CD8 positiven T-Zellen, zur verstärkten Expression des Seneszenzmarkers CD57 und des inhibitorischen Rezeptors LIR-1, sowie in einem Teil der T-Zellen zu einem erhöhten zytotoxischen Potential (Northfield et al. 2005, Pourgheysari et al. 2007, Appay et al. 2008).

Chronische Virusinfektionen wie CMV, aber auch maligne Neoplasien führen zum verstärkten Auftreten von dysfunktionellen, erschöpften $CD8^+$ T-Zellen. Diese T-Zellen sind durch ein komplexes Oberflächenmarkerprofil welches weitestgehend dem TEMRA Differenzierungsstadium (Abb.1) entspricht, den Verlust der Proliferationsfähigkeit und durch funktionelle Inaktivität gekennzeichnet.

Erschöpfte T-Zellen zeigen einige Gemeinsamkeiten mit seneszenten T-Zellen. Der wichtigste Unterschied liegt im Verlust von Haupt-Effektorfunktionen, wie die

Fähigkeit, Zytokine zu sezernieren und Zielzellen zu töten (Akbar and Henson 2011). Seneszente T-Zellen zeigen bisweilen erhöhte Effektorfunktionen, die im Seneszenz-Assoziierten Sekretorischen Phänotyp (SASP) akkumulieren (Callender et al. 2017). Das CMV-spezifische terminal differenzierte T-Zell-Kompartiment beherbergt seneszente und funktionell erschöpfte T-Zellen, deren Ratio einen bedeutenden Einfluss auf die immunologische Gesamtsituation in CMV tragenden Probanden nimmt. Da die Gesamtheit der T-Zellen beschränkt auf den vorhandenen Raum und die Ressourcen ist, sind erhöhte Zahlen an erschöpften T-Zellen für ein intaktes Immunsystem problematisch und werden nicht einfach ersetzt.

1.5. Fragestellung

Innerhalb dieser Habilitation wurden folgende Fragestellungen bezüglich der Rolle von T-Zell-Subpopulationen, T-Zell-Differenzierung und T-Zell-Steuerung bei der rheumatoiden Arthritis, malignen Erkrankungen und der T-Zell-Homöostase untersucht.

T-Zellen spielen für die Pathogenese der rheumatoiden Arthritis und malignen Neoplasien eine herausragende Rolle. T-Zellen sind ursächlich an der Entstehung dieser Erkrankungen beteiligt, bestimmen die Schwere, sind von hoher diagnostischer Relevanz und bilden einen therapeutischen Ansatzpunkt. Das bessere Verständnis T-zellulärer Wirkweisen, krankheitsspezifischer und übergreifender Mechanismen ist daher von hohem medizinischen Interesse.

a) *CMV und T-Zell-Populationen*

Umfangreiche T-zelluläre Untersuchungen an peripherem Blut in Zusammenschau mit dem CMV Status und den klinischen Daten von Patienten/Patientinnen mit rheumatoider Arthritis dienten der Beantwortung der Frage nach dem Zusammenhang zwischen einer CMV Infektion und dem Aufkommen bestimmter T-Zell-Populationen und der Schwere einer rheumatoiden Arthritis.

b) *DP ($CD4^+CD8^+$) T-Zellen*

Zur Identifikation der Bedeutung von $CD4^+CD8^+$ T-Zellen in der rheumatoiden Arthritis wurde peripheres Blut von: i) RA-Patienten/-Patientinnen, ii) SLE Patienten/Patientinnen und iii) gesunden Probanden untersucht. Eine supplementäre Analyse vom Immunzellinfiltrat erfolgte im Synovialgewebe von RA-Patienten/-Patientinnen. Funktionelle Charakterisierungen, *TZR* Repertoirebestimmungen als auch Untersuchungen zur CMV Spezifität sollten Aufschluss über die Pathogenität dieser T-Zell-Subpopulation bei der RA geben.

c) *Kontrolle reaktiver T-Zellen in der Arthritis in vivo*

Zur Beantwortung der Frage nach der T-Zell-Abschaltung bei der Arthritis wurde ein Mausmodell mit einer Spontanmutation im *TZR* verwendet. Diese Tiere entwickeln eine der rheumatoiden Arthritis ähnliche Erkrankung mit steigendem Alter.

Vergleichende Analysen *in vitro* wurden an T-Zellen von Mutanten und Wildtyp-Tieren vorgenommen. In vivo wurde die spontan auftretende Arthritis klinisch und immunzellulär untersucht.

d) *B7-H3 und die T-Zell-Homöostase in der Haut*

In dieser Studie wurde die Bedeutung von Kostimulatormolekülen der Keratinozyten gesunder Haut für die Interaktion mit T-Zellen untersucht. Neben *in situ* Untersuchungen der Haut wurden primäre und immortalisierte Keratinozyten für die Experimente genutzt. T-Zell-Keratinozyten Ko-Kultursysteme zur Identifizierung der Funktion von *B7-H3* komplettierten die Analysen.

e) *T-Zell-inhibitorische B7 Moleküle im Melanom und Nierenzellkarzinom*

Die Rolle des *B7-H4* Moleküls für die Erkrankung des malignen Melanoms wurde mittels *in vitro* Zellsystemen (genetisch manipulierte Melanomzelllinien, *TAA*-spezifische T-Zell-Linien und primäre T-Zellen) und an Patienten-/Patientinnenmaterialien (Histologie und klinische Daten) untersucht.

f) Untersuchungen an Nierenzellkarzinomzelllinien wurden durchgeführt, um die Zytokin-vermittelte Regulation *von B7-H* Molekülen auf Tumorzellen, als Modell eines Tumormikromilieus, zu identifizieren. An der Regulation beteiligte Signalmoleküle wurden durch entsprechende Verfahren ermittelt. T-Zell-Tumor-Kokultursysteme dienten der Beantwortung der Frage nach der funktionellen Konsequenz von zytokingesteuertem *B7-H1* auf Tumorzellen.

2. Ergebnisse/Originalarbeiten

Den hier dargestellten Ergebnissen liegen folgende eigene Originalarbeiten zu Grunde, welche sich im Anhang befinden und die ausgewiesenen Abbildungen beinhalten.

2.1. T-Zell-Subpopulationen mit fundamentaler Bedeutung für den Krankheitsverlauf der rheumatoiden Arthritis

2.1.1. CMV-spezifische T-Zellen und die rheumatoide Arthritis

Pierer M, Rothe K, **Quandt D**, Schulz A, Rossol M, Scholz R, Baerwald C, Wagner U. Association of anticytomegalovirus seropositivity with more severe joint destruction and more frequent joint surgery. Arthritis Rheum. 2012 Jun;64(6):1740-9. **IF: 7,5**

Rothe K. **Quandt D.,** Kristin Schubert[1], Maria Klingner[1], Simon Jasinski-Bergner[2],Roger Scholz[1], MD, Barbara Seliger[2], Pierer M. and Wagner U: Latent CMV infection in RA increases cytolytic LIR-1+ CD8+ T cells, Arthritis Rheumatol. 2016 Feb;68(2):337-46.doi: 10.1002/art.39331 **IF: 7,8**

2.1.2. CD4+CD8+ doppelt positive T-Zellen, die aktiv Einfluss in die Pathogenese der Arthritis nehmen

Quandt D., Rothe K, Scholz R, Baerwald C and Wagner U. Peripheral CD4CD8 doube positive T cells with a distinct helper cytokine profile are increased in rheumatoid arthritis, PLoS One. 2014 Mar 25;9(3):e93293 **IF: 3,2**

2.1.3. Kontrollmechanismen reaktiver T-Zellen in der Arthritis

Rothe K., Raulien N., Pierer M., **Quandt D.** and Wagner U. Autoimmune arthritis induces paired immunoglobulin-like receptor B expression on $CD4^+$ T cells from SKG mice, Eur J Immunol. 2017 Sep;47(9):1457-1467. doi: 10.1002/eji.201646747. Epub 2017 Jul 27., **geteilte Letztautorenschaft** **IF: 4,2**

2.2. Die Bedeutung von Kostimulatoren und Koinhibitoren der B7-Familie für die T-Zell-Homöostase und die antitumorale T-zelluläre Immunabwehr

2.2.1. T-Zell-Steuerung durch B7-H3 auf Hautzellen

Quandt D., Fiedler E, Müller, A, Marsch W Ch and Seliger B.: High constitutive B7-H3 expression on human keratinocytes supports T cell immunity, J Dermatol Sci. 2017 Jul;87(1):82-85. doi: 10.1016/j.jdermsci.2017.02.287. Epub 2017 Apr 11. **IF: 3,7**

2.2.2. T-Zell-Kontrolle durch Koinhibitorexpression auf Tumorzellen

Quandt D., Jasinski, S., Müller U., Schubert, B. and Seliger B. Synergistic effect of IL-4 and TNFα on the induction of B7-H1 in RCC cell lines that inhibits allogeneic T cell proliferation, J Transl Med. 2014 May 30;12(1):151 **IF: 3,9**

Quandt D., Fiedler E, Boettcher D, Marsch W Ch and Seliger B: B7-H4 expression inhuman melanoma: its association with patients' survival and anti tumor immune response Clin Cancer Res. 2011 May 15;17(10):3100-11. Epub 2011 Mar 4 **IF: 7,7**

2.1. T-Zell-Subpopulationen mit fundamentaler Bedeutung für den Krankheitsverlauf der rheumatoiden Arthritis

2.1.1. CMV-spezifische $CD4^+$ und $CD8^+$ T-Zellen und die rheumatoide Arthritis

CMV Infektionen werden oft frühzeitig im Leben erfahren, bleiben latent ein Leben lang erhalten, verlaufen i.d.R. klinisch asymptomatisch, indes hinterlassen sie deutliche Spuren im T-zellulären Kompartiment des Immunsystems (Harari et al. 2004). Inwieweit diese T-zellulären Veränderungen in peripheren $CD4^+$ und $CD8^+$ T-Zellen einen Einfluss auf die Schwere einer rheumatoiden Arthritis haben und welche Moleküle im Besonderen auf T-Zellen differentiell exprimiert werden und funktionelle Konsequenzen nach sich ziehen, wurde in den beiden hier dargestellten Originalarbeiten untersucht.

In einem ersten Arbeitspunkt wurden CMV positive und CMV negative RA-Patienten/-Patientinnen hinsichtlich ihres $CD4^+$ T-Zell-Kompartiments phänotypisch näher charakterisiert. Es wurde eine signifikante Erhöhung $CD4^+CD28^-$ T-Zellen in CMV^+ RA-Patienten/-Patientinnen bei vergleichender Betrachtung zu RA-Patienten/-Patientinnen die CMV negativ sind, gefunden (Abb.1) (Pierer et al. 2012). Eine *in vitro* durchgeführte durchflusszytometrische CMV Peptid-spezifische Proliferationsmessung der T-Zellen zeigte keine verstärkte Proliferation der $CD4^+$ T-Zellen aus RA-Patienten/-Patientinnen gegenüber Kontrollen gesunder Probanden (Abb.1) (Pierer et al. 2012). Der bei einer Proliferation typischerweise verstärkt exprimierte Oberflächenmarker CD71 (Transferrinrezeptor) wurde in den CMV stimulierten Proben aus CMV positiven Probanden hochreguliert. Die Expressionsstärke von CD71 unterschied sich nicht in CMV positiven RA-Patienten/-Patientinnen gegenüber CMV positiven gesunden Kontrollen (Abb.1, (Pierer et al. 2012). Hingegen konnte eine höhere Zahl CMV-spezifischer $CD3^+CD4^+$ *IFN*γ Produzenten in RA gegenüber gesunden Kontrollen (HD) nach *in vitro* Restimulation sowohl mit dem CMV-spezifischen dominanten pp65 Peptid als auch dem CMV Lysat detektiert werden (Abb.2) (Pierer et al. 2012). Der Einsatz des CMV Lysats für die *in vitro* Restimulation der Zellen ermöglicht die Analyse multipler T-Zellen, die spezifisch gegen verschiedene immundominante Epitope des CMV Proteins sind. Durch die notwendige Antigenprozessierung durch Antigen-präsentierende Zellen bei einer *in vitro* Restimulation mittels Protein ist diese Stimulation der *in vivo* Situation ähnlicher und bildet die real existierende Bandbreite verschiedener reaktiver T-Zellen besser ab.

Chemokine sind als Subgruppe der Zytokine wichtige Mediatoren zellulärer Kommunikation. Sie sorgen u.a. für die gerichtete Verteilung von Immunzellen im Körper und im Falle einer Entzündung für die Rekrutierung der Immunzellen an den Entzündungsherd. Chemokin-Spiegel im Serum geben Rückschluss auf einen systemischen Entzündungszustand im Körper. *MCP-1* (*CCL2*) ist vor allem als Chemoattraktor für Monozyten, aber auch für Osteoklasten (Sucur et al. 2017) bekannt. Die Analyse zeigte eine signifikante Erhöhung des Chemokins *MCP-1* in CMV positiven gegenüber CMV negativen RA-Patienten/-Patientinnen (Seite 1744), ((Pierer et al. 2012).

Klinisch konnte festgestellt werden, dass sich RA-Patienten/-Patientinnen mit einer CMV Infektion signifikant öfter einer Synovektomie, bzw. bei additiver Betrachtung von Gelenkersatz und Osteotomie, öfter einer Operation (in der Originalpublikation als „total joint procedures“) im Gelenkbereich unterziehen müssen (Pierer et al. 2012).

Fortführende Untersuchungen zur Rolle CMV-spezifischer $CD8^+$ T-Zellen bei RA-Patienten/-Patientinnen deckten mittels indirekter *TZR* Markierung (Dextramer) einen höheren Prozentsatz CMVpp65 positiver $CD8^+$T-Zellen auf (Abb.1) (Rothe et al. 2016). Andere CMV bekannte T-Zell-Spezifitäten konnten aufgrund technischer Limitationen nicht näher untersucht werden.

Latente CMV Infektionen wurden mit einer erhöhten Expression von *LIR-1* (Immunoglobulin-like transcript 2, CD85j) auf $CD8^+$ T-Zellen assoziiert (Anfossi et al. 2004). LIR-1 wird auf verschiedenen Immunzellen exprimiert und führt i.d.R. mittels eines intrazellulären inhibitorischen Motivs zur Inhibition von Zellen. Die Ergebnisse unserer hier dargestellten Studie zeigen eine verstärkte Frequenz und erhöhte Molekülexpressionsstärke *LIR-1* positiver $CD8^+$ T-Zellen bei CMV positiven RA-Patienten/-Patientinnen im Vergleich zu CMV positiven gesunden Kontrollen. Dieser Befund ist auf das $CD8^+$ T-Zell-Kompartiment begrenzt. $CD4^+$ T-Zellen zeigen im Vergleich zu $CD8^+$ T-Zellen eine 10-fach geringere *LIR-1* Expression, die sich in RA-Patienten/-Patientinnen gegenüber gesunden Kontrollen unverändert darstellt (Abb.1, (Rothe et al. 2016).

Eine vertiefende phänotypische Charakterisierung von LIR-1^+CD8^+ T-Zellen ergab eine Zugehörigkeit zum terminal differenzierten Effektor-Zelltyp mit Verlust von CD27, CD28 und CCR7, verstärkter CD56 Expression und Re-Expression von CD45RA.

PD-1 wurde interessanterweise nicht verstärkt exprimiert (Abb.4), (Rothe et al. 2016). Expressionsanalysen wichtiger Chemokinrezeptoren ergaben eine verstärkte *CX3CR1* Expression auf *LIR-1*$^+$CD8$^+$ T-Zellen von RA-Patienten/-Patientinnen gegenüber gesunden Kontrollen (Abb.4), (Rothe et al. 2016).

Terminal differenzierte proliferativ eingeschränkte T-Zellen nehmen mit dem Alter zu (Akbar et al. 2016). *LIR-1*$^+$CD8$^+$ T-Zellen von RA-Patienten/-Patientinnen korrelieren positiv mit dem Alter (Abb.3), (Rothe et al. 2016). Der größte Unterschied in der Häufigkeit *LIR-1*$^+$CD8$^+$ T-Zellen von RA-Patienten/-Patientinnen gegenüber gesunden Kontrollen ist in der Altersgruppe der unter 50-jährigen Patienten/-Patientinnen feststellbar (Abb.3), (Rothe et al. 2016). Die CMV-spezifische proliferative Kompetenz dieser T-Zellen bleibt unbeeinträchtigt (Abb.5), (Rothe et al. 2016). Untersuchungen zum sekretorischen Phänotyp *LIR-1*$^+$CD8$^+$ T-Zellen ergab funktionell kompetente Zellen, die CMV-spezifisch *IFNγ* produzieren und zytolytische Kompetenz aufweisen. Die Fähigkeit zum CMV-spezifischen Töten zeigt sich bei CD8$^+$ T-Zellen von RA-Patienten/-Patientinnen signifikant erhöht gegenüber gesunden Kontrollen (Abb.5), (Rothe et al. 2016).

HLA-G wurde als Ligand von *LIR-1* beschrieben. Eine *in vitro* vorgenommene Inkubation von HLA-G bei paralleler Aktivierung der *LIR-1*$^+$CD8$^+$ T-Zellen führt zur verminderten *IFNγ* Produktion und geringerer Tötungskompetenz (Abb.6), (Rothe et al. 2016).

Die besondere Rolle der LIR-1$^+$CD8$^+$ T-Zellen in RA-Patienten/-Patientinnen tritt besonders durch die positive Korrelation mit der Krankheitsschwere, ermittelt über den DAS28 (disease activity score), der verschiedene klinische Parameter berücksichtigt, hervor (Abb.3), (Rothe et al. 2016).

Die Ergebnisse der beiden hier vorgestellten Untersuchungen zum T-zellulären CD4$^+$ und CD8$^+$ Kompartiment des Immunsystems in CMV positiven RA-Patienten/-Patientinnen deckten richtungsweisende, zuvor unbekannte krankheitsspezifische Unterschiede sowohl zu CMV negativen RA-Patienten/-Patientinnen als auch zu gesunden CMV positiven Kontrollen auf.

2.1.2. CD4$^+$CD8$^+$ doppelt positive T-Zellen und die Pathogenese der RA

$CD4^+CD8^+$ doppelt positive T-Zellen finden sich im peripheren Blut gesunder Probanden und machen ca. 1% der T-Zellen aus (Parel and Chizzolini 2004). Die Besonderheit liegt in der Expression beider Korezeptoren CD4 und CD8, wobei es sich nicht um T-Zellen der $CD4^+CD8^+$ doppelt positiven Entwicklungsstufe im Thymus handelt. Eine erhöhte Zahl DP T-Zellen mit antiviraler Spezifität und hohem Effektorpotential fand sich nach Virusinfektionen wie HIV und Hepatitis (Nascimbeni et al. 2004, Frahm et al. 2012). Bezüglich einer Beteiligung von DP T-Zellen an Autoimmunerkrankungen zeigt sich eine Arbeit an Patienten/Patientinnen mit systemischer Sklerodermie bedeutungsvoll, bei der diese Zellen erhöht in Hautläsionen gefunden wurden (Parel et al. 2007).

Eine mögliche Rolle der DP T-Zellen für die adulte autoimmune rheumatoide Arthritis war bisher nicht untersucht und hinsichtlich des zuvor dargestellten (2.1.1.) starken Einflusses einer CMV Virusinfektion auf die RA von besonderem Interesse.

Die Untersuchung zur Häufigkeit der DP T-Zellen im peripheren Blut von Patienten/Patientinnen mit rheumatoider Arthritis deckte eine signifikant höhere Zahl dieser Zellen in RA-Patienten/-Patientinnen, die Autoantikörper gegen citrullinierte Peptide tragen (ACPA), im Vergleich zu gesunden Kontrollen gleichen Alters auf (Abb.1), (Quandt et al. 2014). RA-Patienten/-Patientinnen, die keine Autoantikörper gegen citrullinierte Peptide aufweisen, und Patienten/Patientinnen mit SLE wurden vergleichend betrachtet und zeigten keine Erhöhung peripherer $CD4^+CD8^+$ doppelt positiver T-Zellen (Abb.1), (Quandt et al. 2014). Die Anzahl DP positiver T-Zellen in RA-Patienten/-Patientinnen zeigt sich im Gegensatz zu gesunden Kontrollen unabhängig vom Alter.

Phänotypische Untersuchungen peripherer DP T-Zellen in der RA zeigte, dass diese zu über 80% den Gedächtniszellen zugehörig sind (Abb.2), (Quandt et al. 2014). Marker für Differenzierung (CD38) und Aktvierung (HLA-DR) unterschieden sich in ihrer Expressionsstärke signifikant von CD4 einzel positiven T-Zellen (Abb.2), (Quandt et al. 2014). Eine höhere Expression von TCRVa24-Ja18:Vb11 als Marker für iNKT T-Zellen konnte in DP T-Zellen der RA-Patienten/-Patientinnen nicht gefunden werden. Untersuchungen von 24 verschiedenen TZR BV Ketten mittels Durchflusszytometrie deckten keine signifikanten Unterschiede in DP T-Zellen gegenüber CD4 bzw. CD8 einzel positiven T-Zellen in RA-Patienten/-Patientinnen auf (Abb.3), (Quandt et al. 2014).

Zum Verständnis der funktionellen Charakteristika $CD4^+CD8^+$ doppelt positiver T-Zellen in RA-Patienten/-Patientinnen untersuchten wir deren Vorkommen im Synovialgewebe des Gelenks und analysierten das Zytokinprofil dieser Zellen nach einer polyklonalen *in vitro* Restimulation. In Abb. 4 und 5 der Originalpublikation sind die Ergebnisse für die mittels durchflusszytometrischer Analysen gewonnenen Zytokinprofile dargestellt. DP T-Zellen in der RA produzieren mehr *IL-4* und *IFNγ* im Vergleich zu DP T-Zellen aus gesunden Probanden (Quandt et al. 2014). Die Frequenz IL- 21 und IL-17 positiver T-Zellen unterschied sich nicht zwischen DP T-Zellen aus RA-Patienten/-Patientinnen und gesunden Kontrollen. Beim Vergleich der IL-17 Produktion von DP T-Zellen und CD4 einzel positiven T-Zellen aus RA-Patienten/-Patientinnen zeigten sich signifikant erniedrigte Frequenzen IL-17 produzierender DP T-Zellen (Abb.4), (Quandt et al. 2014).

Das pathophysiologische Zielorgan der RA sind die Gelenke. Das Synovialgewebe bildet einen Teil der Gelenkkapsel. Sowohl $CD4^+CD8^+$ doppelt positive T-Zellen als auch B-Zellen wurden nach einem Gewebeverdau im Synovialgewebe der RA-Patienten/-Patientinnen durchflusszytometrisch detektiert (Abb.5), (Quandt et al. 2014).

Ein weiterer wichtiger Untersuchungsschwerpunkt lag in der Betrachtung des Zusammenhangs zwischen einer CMV Infektion und DP T-Zellen in RA-Patienten/-Patientinnen. Es zeigten sich signifikant erhöhte Frequenzen DP T-Zellen in der Peripherie CMV positiver RA-Patienten/-Patientinnen im Vergleich zu CMV negativen RA-Patienten/-Patientinnen (Abb.6), (Quandt et al. 2014). T-Zellen, die den Kostimulator CD28 verloren haben und zu den terminal differenzierten T-Zellen mit polyfunktionellem Potenzial gehören, treten häufiger in CMV positiven RA-Patienten/-Patientinnen auf (Pierer et al. 2012). Die Frequenz DP T-Zellen korreliert positiv mit der Frequenz an $CD4^+CD28^-$ Zellen in RA-Patienten/-Patientinnen (Abb.6), (Quandt et al. 2014). Besonders wichtig ist der Befund, dass DP T-Zellen von RA-Patienten/-Patientinnen eine CMV-spezifisch erhöhte Produktion an inflammatorischem Effektorzytokin *IFNγ* im Vergleich zu gesunden Kontrollen aufwiesen (Abb.6), (Quandt et al. 2014).

Zusammenfassend stellen die Ergebnisse dieser Studie die Pathogenität peripherer $CD4^+CD8^+$ doppelt positiver T-Zellen, insbesondere bei Vorhandensein von Autoantikörpern (ACPA+) und CMV positiver rheumatoider Arthritis dar.

2.1.3. Kontrollmechanismen reaktiver T-Zellen in der Arthritis

Mausmodelle unterstützen wesentlich den Erkenntnisgewinn zur Entstehung, zum Progress, der Zellbeteiligung und den Therapieoptionen verschiedenster humaner Krankheiten. Zahlreiche Mausmodelle für die Arthritis, beispielsweise die Kollagen-induzierte Arthritis (CIA), die TZR transgene K/BxN Maus, das TNFα-Transgen Modell und die Arthritis im SKG Mausmodell trugen und tragen zum vorhandenen Erkenntnisstand der pathogenen Prozesse der humanen rheumatoiden Arthritis bei (Bessis et al. 2017).

Die SKG Arthritis in Balb/c Tieren geht auf eine Spontanmutation in der SH2C Domaine der zeta- Ketten assoziierten Proteinkinase 70 (ZAP-70) des T-Zell-Rezeptorkomplexes zurück. Diese genetische führt zu einer veränderten T-Zell-Aktivierung (Sakaguchi et al. 2003). Es kommt zur positiven Selektion autoreaktiver T-Zell-Klone im Thymus. Diese T-Zellen sind im Zusammenspiel mit dem Mikrobiom (Yoshitomi et al. 2005) hauptverantwortlich für die Entwicklung einer spontanen Arthritis mit entzündlichen Gelenkschwellungen.

PIR-B ist das dem humanen LIR-1 orthologe Molekül im Mausmodell. Die bisherige Literatur weist *PIR-B* vor allem in B-Zellen, dendritischen Zellen, Mastzellen und präthymischen Progenitoren, nicht in reifen T-Zellen von Wildtyp-Tieren aus (Kubagawa et al. 1999, Imada et al. 2009).
Die Ergebnisse der hier durchgeführten durchflusszytometrischen Untersuchung zeigen erstmals eine konstitutive und altersabhängige Expression von *PIR-B* in $CD4^+$ und $CD8^+$ T-Zellen in sekundär lymphatischen Organen in SKG Tieren (Abb.1 und Daten nicht gezeigt), (Rothe et al. 2017). Mäuse anderer, oft für die experimentelle Induktion einer Arthritis genutzten Stämme (DBA/J und BL/6) als auch Balb/c Tiere zeigten altersunabhängig keine bzw. eine geringe (Balb/c, auf Milz beschränkt) Expression von *PIR-B* (Daten nicht gezeigt und (Abb.1), (Rothe et al. 2017). Das Transkript für *PIR-B* konnte mittels konventioneller RT-PCR nachgewiesen werden. Ein extrem wichtiger Befund der einen Molekültransfer von stark *PIR-B* exprimierenden Zellen unwahrscheinlich macht (Supp-Abb.1), (Rothe et al. 2017). Beachtlich, die Expressionsstärke von *PIR-B* auf CD4+ T-Zellen korreliert mit dem Mikrobiom. Tiere in konventioneller Haltung wiesen eine stärkere Expression von *PIR-B* auf im Vergleich zu Tieren unter spezifisch pathogenfreien (SPF) Haltungsbedingungen.

Die phänotypische Charakterisierung der *PIR-B*$^+$ T-Zellen zeigte eine erhöhte Expression des Aktivierungsmarkers CD69 und des Koinhibitors *PD-1* (Abb.1), (Rothe et al. 2017). Zur Beantwortung der Frage nach funktionellen Unterschieden von *PIR-B*$^+$ T-Zellen wurden Zytokine mittels durchflusszytometrischen Zytokinsekretionsassays bzw. intrazellulären Zytokinnachweises bestimmt. *PIR-B*$^+$ T-Zellen aus SKG Tieren zeigten signifikant höhere *IFNγ* und *IL-17* Produktion im Vergleich zu *PIR-B* negativen T-Zellen nach *in vitro* Restimulation mit *PMA*/Ionomycin (Abb.4), (Rothe et al. 2017). *IL-17* zählt zu den Zytokinen mit dem größten Einfluss auf das inflammatorische Milieu und infolge dessen auf das destruktive Geschehen bei der Arthritis. *PIR-B* positive T-Zellen sezernieren bemerkenswerterweise bis zu 5x mehr *IFNγ* im Vergleich zu *IL-17*. *PMA*/Ionomycin wurde als Stimulus gewählt, da bekannt war, dass T-Zellen aus SKG Tieren eine geringere Aktivierbarkeit durch *TZR* Kreuzvernetzung aufweisen (Hirota et al. 2007). Dieser Unterschied in der Aktivierbarkeit von T-Zellen aus SKG Tieren durch *TZR* Kreuzvernetzung mittels Antikörpern gegen CD3 bzw. durch PMA/Ionomycin (direkter Trigger von *PKC* und Ca^{2+}-Freisetzung) konnte mittels der hier erhobenen Daten bestätigt werden (Abb.2 und 3), (Rothe et al. 2017). *In vitro* Aktivierung von T-Zellen aus SKG und Balb/c Tieren führt zur Induktion von *PIR-B*, wobei SKG T-Zellen stets eine stärkere Expression zeigten (Abb.2 und 3), (Rothe et al. 2017). Die Aktivierung kann dabei sowohl über ein *TZR* Signal als auch über einen Trigger der *PKC* mit Calcium-Mobilisierung erfolgen. Die *in vitro* induzierten *PIR-B* exprimierenden Zellen aus Balb/c Tieren sezernieren *IFNγ*, nicht aber *IL-17*. *PIR-B* induziert daher nicht die *IL-17* Produktion, sondern *IL-17* Produzenten aus arthritischen SKG Tieren exprimieren verstärkt *PIR-B*.

Eine hohe Expression von PIR-B zusammen mit einem inflammatorischen Zytokinprofil in 11-Monate alten SKG Tieren geht einher mit einer klinisch messbaren Arthritis. Die Arthritis ist zum diesem Zeitpunkt bereits manifestiert, erste klinische Anzeichen wurden im Alter von 4-6 Monaten dokumentiert. SKG Tiere mit einer geringeren *PIR-B* Expression (<35%) haben im Vergleich zu Tieren mit einer *PIR-B* Expression größer als 35% einen höheren Arthritis-Score, welcher durch die Stärke und Anzahl geschwollener Pfoten bestimmt wird (Abb.6), (Rothe et al. 2017). Diese Korrelation einer hohen Zahl an *PIR-B* positiven CD4 T-Zellen mit einer milderen Arthritis konnte zusätzlich durch einen histologischen Arthritis-Score als Maß der Zellinfiltration und Knorpeldestruktion bestätigt werden (Abb.6), (Rothe et al. 2017).

PIR-B positive T-Zellen in der Arthritis sind zusammenfassend betrachtet nicht die treibende Kraft eines destruktiven Krankheitsverlaufes, sondern *PIR-B* fungiert eher als attenuierendes Signal. Die Hypothese einer Aktivierungsbegrenzung über *PIR-B* wird durch eine dominierende *IFN*γ Produktion und durch erhöhte *PD-1* (koinhibitorischer Rezeptor) Expressionsspiegel unterstützt.

2.2. Die Rolle von Kostimulatoren und Koinhibitoren für die T-Zell-Homöostase und die antitumorale T-zelluläre Immunabwehr

2.2.1. T-Zell-Steuerung durch B7-H3 auf Hautzellen

Die Haut beherbergt im Vergleich zum peripheren Blut die doppelte Zahl an T-Zellen (Clark 2010). Diese gehören meist den Gedächtnis-T-Zellen an und sind sowohl vom CD4 T-Helferzelltypus als auch vom CD8 T-Zell-Typ, wobei insbesondere kaum zytolytische Potenz von diesen T-Zellen ausgeht (Schaerli et al. 2004, Judith A. Seidel 2016). Keratinozyten der Haut interagieren mit $CD4^+$ und $CD8^+$ T-Zellen, wobei durch eine fehlende konstitutive Expression von *HLA* Klasse II Molekülen die Interaktion mit CD4 T-Zellen erst im entzündlichen Prozess möglich wird (Griffiths et al. 1990). *B7-H3* gehört zur *B7-H* Molekül-Familie der Kostimulatoren/Koinhibitoren, für das 2 unterschiedliche Rezeptoren auf T-Zellen vermutet werden, welche durch kontroverse Forschungsergebnisse noch nicht abschließend anerkannt sind (Hashiguchi et al. 2008, Leitner et al. 2009).

Die Untersuchungen dieser hier vorgestellten Studie deckten eine konstitutiv hohe Expression von *B7-H3 in situ* in gesunden Keratinozyten der Basalzellschicht der Epidermis auf (Abb.1), (Quandt et al. 2017). Diese *B7-H3* Expression war auf 80% der Biopsien oberflächlich und intrazellulär detektierbar. Die Stärke der Molekülexpression war von einer angrenzenden malignen oder benignen Hautpartie unabhängig (Supplement (Quandt et al. 2017).

Anschließende *in vitro* Experimente an primären und immortalisierten Keratinozyten bestätigten eine hohe konstitutive Expression von *B7-H3* auf Transkript- und Proteinebene (Abb.1), (Quandt et al. 2017). INFγ Behandlung von Keratinozyten verändert die *B7-H3* Expression nicht, wobei andere Moleküle der *B7* Familie, der Adhäsion- und Antigenpräsentation, wie *B7-H1, ICAM-1* und *HLA*-Klasse-II signifikant hochreguliert wurden (Abb.1), (Quandt et al. 2017).

Zur Bestimmung der Rolle von *B7-H3* auf Keratinozyten für die T-Zell-Immunologie, wurde *B7-H3* experimentell mittels spezifischer shRNA herunterreguliert (Abb.2, (Quandt et al. 2017), wobei *B7-H1* und *HLA*-Klasse-I Moleküle unverändert exprimiert blieben. Ko-Kulturen von T-Zellen gesunder Spender mit den *B7-H3* manipulierten Keratinozyten wurden durchgeführt. Das Prinzip einer alloreaktiven T-Zell-Antwort kam zur Anwendung, bei der die T-Zellen aus Spendern mit einem zu

den Keratinozyten (*HLA*-A31,-B40,-B51) divergenten *HLA*-A,-B Typ ausgestattet waren. T-Zell-Proliferation, T-Zell-Aktivierung und T-zelluläre *IFNγ* Produktion zeigten sich nach Ko-Kultur mit herunterreguliertem *B7-H3* auf Keratinozyten verringert (Abb.2, (Quandt et al. 2017).

Die Ergebnisse der *B7-H3* Studie deckten ein völlig neues Expressionsprofil des Moleküls auf humanen Keratinozyten auf, welches nicht durch eine maligne Neoplasie gesteuert wird. Funktionell konnte eine kostimulatorische Funktion für T-Zellen identifiziert werden, welche sich in vivo als Teil der homöostatischen Erhaltung der T-Zellen in der Haut darstellen könnte.

2.2.2. T-Zell-Kontrolle durch Koinhibitorexpression auf Tumorzellen

Moleküle der Familie der Kostimulatoren stellen einen komplexen Regulationsmechanismus zur Steuerung von T-Zell-Immunantworten dar (Seliger and Quandt 2012).

Zu Beginn dieser Untersuchungen war bekannt, dass Tumorzellen Moleküle verschiedener Kostimulatorfamilien, so auch der B7-Familie, exprimieren können (Schildberg et al. 2016). *B7-H1* (*PD-L1*) *B7-H4* wurden als Inhibitor der T-Zell-Antworten in verschiedenen Tumorentitäten beschrieben und konnte mit klinischen Parametern der Patienten/Patientinnen assoziiert werden (Krambeck et al. 2006, Thompson et al. 2006, Hino et al. 2010). Die Bedeutung von *B7-H4* für das maligne Melanom war bis dahin noch unverstanden. Die Regulation der Expression von *B-H1* und *B7-H4* auf Tumorzellen ist für das Verständnis ihrer Rolle in der Tumorpathogenese und ihrer therapeutischen Manipulierbarkeit essentiell. Inflammatorische Zytokine der Familie der Interferone steuern die Expressionsstärke von *B7-H1* (Blank et al. 2005, Kondo et al. 2010). Die Rolle von anti-inflammatorischen Interleukinen und inflammatorischen Zytokinen der TNF Familie für die Expression und funktionelle Konsequenz von *B7-H1,-H2, -H3*, und *B7-H4* auf Nierenzellkarzinomzellen war ebenfalls noch ungeklärt und stellte daher einen wichtigen Arbeitspunkt dar.

Es konnte in den durchgeführten Untersuchungen *B7-H4 in situ* in Melanomzellen von Patienten/Patientinnen mit malignem Melanom gefunden werden (Abb. 1), (Quandt et al. 2011). Die Expressionsstärke von *B7-H4* lag im Primärtumor und den

Metastasen signifikant über der des angrenzenden gesunden Hautgewebes. Die Expression zeigte sich des Weiteren in der Patienten-/Patientinnenkohorte unterschiedlich, womit eine Gruppierung in *B7-H4* niedrig- und hochexprimierend indiziert war (Abb. 1), (Quandt et al. 2011). Das Immunzellinfiltrat der malignen Melanome bestand vor allem aus $CD8^+$ T-Zellen und $CD68^+$ Makrophagen, wobei letztere signifikant erhöht in *B7-H4* hochexprimierenden Patienten/Patientinnen gefunden wurden (Tab. 1), (Quandt et al. 2011)). Von besonderer Bedeutung ist der Befund, dass eine niedrigere *B7-H4* Expression mit einem längeren Überleben assoziiert war (Abb. 2), (Quandt et al. 2011). Umfangreiche Untersuchungen an Zelllinien zeigten trotz positiver Transkripte für *B7-H4* keine konstitutive Proteinexpression auf Melanomzelllinien, wohingegen Brustkrebstumorzelllinien für *B7-H4* positiv durchflusszytometrisch markiert wurden (Abb.3), (Quandt et al. 2011). Experimentelle Überexpression von *B7-H4* und anschließende T-Zell-Tumor Kokulturassays zeigten keine Veränderung für die T-zelluläre Tötungskompetenz, aber gleichwertig relevant konnte eine Inhibition der T-zellulären Zytokinproduktion von *IFNγ, IL-2* und *TNFα* festgestellt werden. Dies galt für Melan-A monospezifische als auch für multispezifische T-Zellen gesunder Spender, welche durch wiederholte Restimulationen mit Tumorzellen generiert wurden (Abb.5), (Quandt et al. 2011). *B7-H3* wurde in Melanomen *in situ* und in den Melanomzelllinien *in vitro* konstitutiv exprimiert, wobei sich aber keine Assoziation zu klinischen Daten finden ließ und melanomspezifische T-Zellen keine unterschiedlichen Effektorfunktionen nach experimenteller Blockade der *B7-H3* Expression auf den Tumorzellen zeigten (Abb.4), (Quandt et al. 2011).

Die Behandlung von verschiedenen Nierenzellkarzinomzelllinien mit *IL-4, TNFα* oder der Kombination beider Zytokine führte zu einer unterschiedlichen Regulation von *B7-H1-B7-H4* Molekülen. *B7-H1* wird durch singuläre Gabe von *IL-4* und *TNFα* zeitabhängig auf mRNA und Proteinebene reguliert, wobei die Kombination beider Zytokine zu einer synergistischen Hochregulation führt (Abb.2 und 3), (Quandt et al. 2014). *B7-H2* wird durch TNFα hochreguliert, wobei die Addition von *IL-4* keinen zusätzlichen Effekt brachte. *B7-H3* und *B7-H4* blieben in ihrer Expression von den Zytokinen als Einzelgabe oder in Kombination weitestgehend unbeeinflusst. Nierenzellkarzinomzelllinien führen kein Transkript für *IL-4*, wohingegen *TNFα* als Transkript vorliegt, aber als Protein nur in wenigen Zelllinien in sehr geringen Dosen von 40pg/ml sezerniert wird (Abb.1), (Quandt et al. 2014).

Die Zytokin-induzierte Hochregulation von *B7-H1* führt in allogenen *PD-1* tragenden T-Zell-Tumor-Kokulturen zu einer T-Zell-Proliferationsblockade, wobei die Sekretion von *IFNγ* unbeeinflusst blieb (Abb.5), (Quandt et al. 2014).
Als beteiligte Signalmoleküle wurden *NFkB*, *IkB* und *STAT6* ermittelt, deren Phosphorylierungsmuster sich verändert zeigten, wobei NFKB am Serin 529 deutlich stärker durch die Kombination beider Zytokine phosphoryliert wird als durch TNFα allein und *IL-4* keinen Einfluss auf die Phosphorylierung von *NFkB* nimmt (Abb.4), (Quandt et al. 2014). Die Kombination beider Zytokine war mit einer deutlich gesteigerten Promotoraktivität von *B7-H1* verbunden. Die Microarray-Daten einer frei verfügbaren Datenbank zeigten eine positive Korrelation von *B7-H1* mit *NFkB* und *STAT6* in Nierentumorgeweben (Abb. 4), (Quandt et al. 2014) und r2 database, (http://r2.amc.nl).

Zusammenfassend komplettieren und erweitern diese beiden Arbeiten die Kenntnisse über inhibitorische Moleküle der *B7* Familie auf Tumoren. *B7-H4* könnte für die Therapie des malignen Melanoms eine neue Zielstruktur bilden. Die Erkenntnisse zu *B7-H1* sind im besonderen aktuell von hoher Relevanz, da die *PD-L1* Antikörpertherapie als neue Immun-Checkpoint-Therapie für das maligne Melanom, das Nierenzellkarzinom und andere Tumorentitäten auf dem Vormarsch ist.

3. Diskussion/Ausblick

3.1. T-Zell-Subpopulationen mit fundamentaler Bedeutung für den Krankheitsverlauf der rheumatoiden Arthritis

3.1.1. CMV Virus-spezifische CD4$^+$ und CD8$^+$ T-Zellen, LIR-1 und die rheumatoide Arthritis

CMV Infektionen hinterlassen deutliche Spuren in T-zellulären aber auch in Natürlichen Killerzellen (Heath et al. 2016) Kompartimenten des adaptiven/angeborenen Immunsystems in gesunden Probanden. Es kommt zu einem differenzierten T-zellulären Phänotyp mit erhöhtem Verlust des Kostimulators CD28 auf CD4 und CD8 positiven T-Zellen, zur verstärkten Expression des Seneszenzmarkers CD57 und des inhibitorischen Rezeptors *LIR-1*, sowie zu einem erhöhten zytotoxischen Potential (Northfield et al. 2005, Pourgheysari et al. 2007, Appay et al. 2008). Diese CMV-spezifischen T-zellulären Veränderungen konnten in unserer Studie in der gesunden Kontrollkohorte bestätigt werden.
Die Daten der beiden hier vorgestellten Originalarbeiten zeigen, dass diese T-zellulären Veränderungen durch eine rheumatische Erkrankung ausgebaut werden. Es konnten erhöhte Zahlen von CD4$^+$CD28$^-$ T-Zellen mit gesteigerter Kapazität *IFNγ* zu produzieren und erhöhte Zahlen von LIR-1$^+$CD8$^+$ T-Zellen mit einem verstärkten zytotoxischen Phänotyp, die mit der Krankheitsschwere korrelieren, identifiziert werden. Diese T-zellulären Veränderungen gehen mit einer gesteigerten Inzidenz an operativen Eingriffen bei CMV positiven RA-Patienten/-Patientinnen einher (Abb.5, Diskussion).

Es wird vermutet, dass eine persistente CMV-Infektion zur klonalen Expansion von funktionell erschöpften (*exhausted*) T-Zellen führt. Suboptimal agierende CMV-spezifische T-Zellen hätten zur Folge, dass mehr T-Zellen für die Kontrolle der CMV-Infektion benötigt würden (Akbar and Fletcher 2005).

Beachtlich, dysfunktionelle (exhausted) T-Zellen werden infolge systemischer chronischer Viruserkrankungen wie beispielsweise CMV als auch in Tumor infiltrierenden T-Zell-Populationen verschiedener Tumorentitäten gefunden. Das Vorhandensein inhibitorischer Moleküle wie *LIR-1, PD-1, TIGIT, BTLA* oder auch *CTLA-4,* die mit dem dysfunktionellen Phänotyp bei Virus- und Tumorerkrankungen assoziiert werden (Wherry and Kurachi 2015), kann nicht allein Aufschluss über die Zugehörigkeit der T-Zell-Populationen geben, da sie ebenfalls im Laufe einer gesunden T-Zell-Aktivierung differentiell exprimiert werden. Dysfunktionelle T-Zellen

zeigen zusätzlich zu der Kombination inhibitorischer Oberflächenmoleküle eine verminderte Proliferation auf. Darüber hinaus weisen sie typische von anderen T-Zell-Populationen distinkte Transkriptomprofile und epigenetische Signaturen auf (Wang et al. 2017), die Zielstrukturen therapeutischer Interventionen bilden. *LIR-1*$^+$ CD8$^+$ T-Zellen unserer Studie zeigen eine CMV-spezifisch gesteigerte Proliferation gegenüber *LIR-1*$^-$ CD8$^+$ T-Zellen, was gegen eine dysfunktionelle Subpopulation spricht. Eine kürzlich erschienene Arbeit bestätigt die Zugehörigkeit der *LIR-1*$^+$ CD8$^+$ T-Zellen zur Population mit seneszentem Phänotyp. Untypisch für seneszente T-Zellen sind sie aber mit einem hohen CMV-spezifischen proliferativem und zytolytischem Effektorpotential in gesunden Probanden ausgestattet (Gustafson et al. 2017).

Der Befund der erhöhten Expression des Fraktalkin-Rezeptors CX3CR1 auf LIR-1$^+$ CD8$^+$ T-Zellen in RA impliziert eine erhöhte Mobilität dieser T-Zell-Population. Fraktalkinspiegel im Serum als auch im Synovium sind bei RA-Patienten/-Patientinnen erhöht, wodurch *CX3CR1* tragende Monozyten, Osteklasten und T-Zellen angelockt, aktiviert und ihr Überleben gesteuert wird (Nanki et al. 2017). *LIR-1*$^+$ CD8$^+$ T-Zellen konnten in unseren Untersuchungen ebenfalls in der Synovialflüssigkeit und der Synovialmembran detektiert werden (Daten nicht gezeigt) und sind möglicherweise über eine Fraktalkin-spezifische Migration eingewandert.

LIR-1 bindet verschiedene klassische (*HLA-A* und *HLA-B*) und nicht-klassische *HLA* Klasse I Moleküle wie *HLA-G*, wobei letzteres mit deutlich stärkerer Bindungsaffinität (Shiroishi et al. 2003).

HLA-G ist weitläufig als Molekül der embryonalen Immuntoleranz und als Tumor-Escape-Mechanismus bekannt (Seliger 2016). In Patienten/Patientinnen mit rheumatischen Erkrankungen wurden zum einen verminderte lösliche Serum-Spiegel für *HLA-G* gemessen (Verbruggen et al. 2006), zum anderen wurde in einer aktuelleren Studie eine verminderte Bindungsaffinität von löslichem *HLA-G* für *LIR-1* (Veit et al. 2015) dokumentiert. Beide Studien über die geringere Bioverfügbarkeit von löslichem *HLA-G* lassen sich gut mit unserem Ergebnis einer reaktiveren unkontrollierten Effektorpotenz der *LIR-1*$^+$CD8$^+$ T-Zellen in RA-Patienten/-Patientinnen korrelieren. Die erhöhte *LIR-1* Expression der CD8$^+$ T-Zellen bei RA könnte somit ein Mechanismus der versuchten Immunkontrolle dieser T-Zell-Population sein. Diese Hypothese stützt sich auf die hier vorliegenden Ergebnisse, dass die zytotoxische Aktivität der *LIR-1*$^+$ T-Zellen durch eine *in vitro* Gabe von

löslichem *HLA-G* hemmbar ist, somit *LIR-1* funktionell auf den $CD8^+$ T-Zellen der RA-Patienten/-Patientinnen vorliegt. Gustafson et al. beschreibt bei gesunden Spendern eine gesteigerte CMV-spezifische Proliferation von *LIR-1*$^+$ T-Zellen bei unveränderter Zytokinproduktion in einem *in vitro* Zellsystem, bei dem *LIR-1* durch Antikörper blockiert ist und somit seine inhibitorische Wirkung vermittelt durch *HLA* Klasse I nicht wahrnehmen kann (Gustafson et al. 2017). Die Diskrepanz bezüglich der CMV-spezifischen Zytokinproduktion zu unseren Daten könnte an den unterschiedlichen *in vitro* Systemen zur Identifizierung der funktionellen Kompetenz *LIR-1*$^+$ T-Zellen liegen und/oder dem Spenderkollektiv gesunder Probanden (Gustafson et al.) gegenüber RA-Patienten/-Patientinnen (Rothe, Quandt et al.) geschuldet sein.

Gemeinsam weisen die Ergebnisse aus der in dieser Schrift dargelegten Studie und der von Gustafson et al. auf eine Immun-Checkpoint Funktion von *LIR-1* hin, welche bei entsprechender Bindungspartnerverfügbarkeit die Größe des CMV-spezifischen Effektorpools kontrolliert und die zytolytische Effektorpotenz CMV-spezifischer Zellen bei RA-Patienten/-Patientinnen eingrenzen kann.

In CMV negativen RA-Patienten/-Patientinnen scheint die chronische inflammatorische Situation ebenfalls zur Verstärkung des *LIR-1* Moleküls auf $CD8^+$ T-Zellen beizutragen, obwohl die Ergebnisse hier nur Tendenzen zeigten (Rothe et al. 2016). Diese Befunde unterstützen eine mögliche, eher generelle Aufgabe des *LIR-1* Moleküls in der T-Zell-Biologie bei entzündlichen chronischen Prozessen, zu denen letztlich auch die Situation einer Tumorerkrankung zählt.

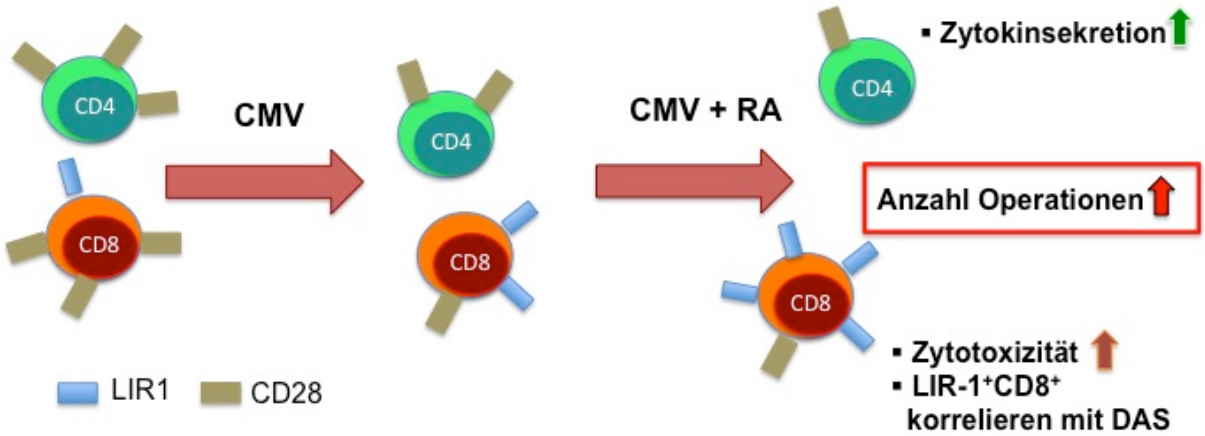

Abb.5 T-zelluläre Veränderungen durch CMV und rheumatoide Arthritis,
CMV: Cytomegalovirus, DAS: disease activity score, RA: rheumatoide Arthritis

Eine natürliche CMV Infektion führt zur Aktivierung klassischer *HLA*-Klasse I-restringierter CD8 T-Zell-Antworten. Bemerkenswerte neue Forschungsansätze in der Impfstoffentwicklung zeigen den Einsatz von CMV basierten, genetisch veränderten Vektoren, welche klassische und nicht-klassische (*HLA-II* restringiert und *HLA-E* restringierte) CD8 T-Zell-Antworten in Primaten hervorrufen (Fruh and Picker 2017). Eine Impfung gegen CMV, bzw. gegen andere bisher nicht-kontrollierbare Viren, könnte erfolgreich sein, wenn die natürlich auftretende insuffiziente CD8 T-Zellantwort um eine vielgestaltige CD8 T-Zell-Antwort mit hoher Zahl an potenten Effektor-Gedächtnis-Zellen erweitert würde, die keine systemischen T-zellulären Veränderungen, wie oben ausgeführt, hinterlassen.

3.1.2. Polyfunktionale $CD4^+CD8^+$ doppelt positive T-Zellen in der Pathogenese der RA

Die Ergebnisse dieser Studie zeigen zum ersten Mal erhöhte polyfunktionale $CD4^+CD8^+$ doppelt positive T-Zellen in der Peripherie von adulten $ACPA^+$ RA-Patienten/-Patientinnen. In einer früheren Untersuchung an 4 Patienten mit juveniler rheumatoider Arthritis konnten $CD4^+CD8^+$ doppelt positive T-Zellen aus dem Synovialgewebe isoliert werden, Untersuchungen in der Peripherie und zur Funktion wurden nicht durchgeführt (De Maria et al. 1987).

$CD4^+CD8^+$ doppelt positive T-Zellen lassen sich hinsichtlich der Intensität der Ko-Rezeptorexpression in 3 weitere Subgruppierungen ($CD4^{++}CD8^+$, $CD4^{++}CD8^{++}$, $CD4^+CD8^{++}$) unterteilen (Parel and Chizzolini 2004, Overgaard et al. 2015). Die hier erhobenen Daten zeigen die am häufigsten vertretene Fraktion in der Subgruppe der $CD4^{++}CD8^+$ positiven T-Zellen. Diese Tatsache, zusammen mit publizierten Daten, die eine Blockade des CD4 typischen Transkriptionsfaktorprogramms, das für eine *de novo* CD8 Expression sorgt (Mucida et al. 2013, Reis et al. 2013), lässt die Vermutung zu, dass diese Zellen aus peripheren CD4 einzel positiven T-Zellen entstanden sind. Der Fakt, dass DP T-Zellen in der RA erhöhte Mengen an *IL-4* sezernieren, könnte für die Aufrechterhaltung der CD8 Expression von DP T-Zellen mitverantwortlich sein, denn *IL-4* wurde in einer früheren Arbeit als Triebkraft für eine CD8 Expression identifiziert (Paliard et al. 1988).

Die Ergebnisse dieser Studie zeigen eine erhöhte Zahl DP T-Zellen in Patienten/Patientinnen mit Antikörpern gegen citrullinierte Peptide. T-Zell-Antworten gegen citrullinierte Peptide aus Proteinen des Zytoskeletts, der Blutgerinnung und

der Hautverhornung wurden bei RA gefunden (Snir et al. 2011, Gertel et al. 2017). Eigene Ergebnisse an CD4 einzel positiven T-Zellen von RA-Patienten/-Patientinnen zeigen erhöhte Zytokinproduzenten beim Kontakt mit citrulliniertem Vimentin gegenüber nicht-citrulliniertem Vimentin (Daten nicht gezeigt).
Fortführende Untersuchungen zur Spezifität von DP T-Zellen für citrullinierte Peptide aus Proteinen wie Vimentin, Fibrinogen und Fillagrin, den Hauptautoantigenen der RA (Masson-Bessiere et al. 2001, Vossenaar et al. 2004) könnten sich sinnvoll an die hier gewonnen Erkenntnisse anschließen.
Die T-Zell-Antwort bei der RA weist vor allem im Verlauf einen klaren *Th1* Bias mit vorrangiger Produktion von *IFNγ* und *IL-17* auf (Leipe et al. 2010). Interessanterweise hängt das Zytokinprofil im Synovium vom Zeitpunkt der Untersuchung ab. So liegt in RA-Patienten/-Patientinnen in einem frühen Stadium eher ein Th2 Phänotyp mit erhöhtem Level an IL-4 und IL-13 und kaum *IFNγ* in der Synovialflüssigkeit vor (Raza et al. 2005). Das Vorhandensein von IL-4 Produzenten bei der RA wird auch von Sieper et al., 1999 nach polyklonaler Restimulation der Zellen *in vitro* bestätigt (Yin et al. 1999). Eine weitere Arbeit zeigt, dass synoviales *IL-4* von CD8 T-Zellen produziert wird (Cho et al. 2012). Diese CD8 T-Zellen könnten gleichzeitig CD4 positiv sein, was in der Studie von Cho et al. nicht betrachtet wurde. Darüber hinaus kann *IL-4* entscheidend in die pathogene Antikörperproduktion bei der RA, zumindest in einem der humanen RA sehr ähnlichem Mausmodell, eingreifen (Ohmura et al. 2005).

Die Ergebnisse unserer Untersuchungen weisen des Weiteren auf eine Multifunktionalität der DP T-Zellen bei der RA hin. Neben den *IL-4* Produzenten sezernieren diese T-Zellen ebenfalls CMV-spezifisch mehr *IFNγ* als die Vergleichspopulation aus gesunden Spendern. Eine latente CMV Infektion führt, wie zuvor ausgeführt (3.1.1.), zum schwereren Verlauf einer rheumatoiden Erkrankung. Dies könnte in der höheren Zahl reaktiver T-Zellen, die CD4 und CD8 einzel positiv, aber auch $CD4^+CD8^+$ doppelt positiv sind, begründet sein. DP positive T-Zellen konnten bei gesunden Spendern bereits mit einer CMV Infektion korreliert werden. Diese T-Zellen zeigten eine CMV-spezifische verstärkte Zytokinproduktion, Proliferation und Zytotoxizität (Suni et al. 2001). Kürzlich wurden diese Befunde für DP T-Zellen aus gesunden Spendern mit einer vorliegenden CMV Reaktivität erweitert. T-bet und Eomes, zwei wichtige Transkriptionsfaktoren, die zahlreiche T-Zell-Effektorfunktionen steuern, sind in CMV+ gegenüber CMV- DP T-Zellen und

gegenüber CD4 bzw. CD8 einzel positiven Zellen erhöht exprimiert (Hassouneh et al. 2017).

In Zusammenschau mit zahlreichen anderen Studien der letzten 25 Jahre, bestätigt unsere Studie die Existenz peripherer $CD4^+CD8^+$ doppelt positiver T-Zellen mit krankheitsspezifischem Effektorpotenzial (Overgaard et al. 2015). Dennoch ist es wissenswert, dass die genaue Anzahl dieser Zellen vermutlich geringer ist als vielfach publiziert, da es methodisch bedingt obgleich wie in unserer Studie angewandter state of the art durchgeführter durchflusszytometrischer Analytik, zur Detektion einer geringen Anzahl (≤ 0,3%) von $CD4^+CD8^+$ Zellaggregaten kommt *(Overgaard et al. 2017).*

Mehr und mehr in den Fokus der Untersuchungen rückt die Rolle des Metabolismus für T-Zell-Funktionen bei der rheumatoiden Arthritis. Arthritogene T-Zellen wechseln für ihren Energiehaushalt von der Glykolyse zum Pentosephosphatweg. Dies ist begleitet von einem reduzierten Redox-Signaling, was sich in Hyperproliferation, einem veränderten Zellzyklus und Zeichen einer vorzeitigen Alterung niederschlägt (Weyand et al. 2017). Inwieweit $CD4^+CD8^+$ doppelt positive T-Zellen diesen veränderten Metabolismus aufzeigen ist noch unverstanden und wird Gegenstand fortführender Untersuchungen sein.

Von vermutlich fundamentaler Bedeutung für das Verständnis der Entstehung und den Verlauf der rheumatoiden Arthritis ist die in den letzten Jahren verstärkt erforschte Rolle des Mikrobioms. Als Mikrobiom wird die Gesamtheit der vorhandenen Mikroorganismen eines Organismus bezeichnet. Sowohl das Darm- als auch das Mundmilieu-assoziierte Mikrobiom nehmen Einfluss auf den Verlauf der RA (Lee and Kim 2017). Das mit einer Periodontitis assoziierte Bakterium Porphyromonas gingivalis kann für eine verstärkte *TH17* Polarisierung sorgen und darüber hinaus die Citrullinierung von Proteinen ermöglichen (Rosenstein et al. 2004, Moutsopoulos et al. 2012). Beide Vorgänge sind essentiell wichtig für den Progress bzw. die Entstehung von RA.
DP T-Zellen sind aktive Gestalter der Pathogenese bei RA und bilden sowohl neue Zielstrukturen in der Diagnostik als auch für neue therapeutische Ansätze.

3.1.3. Kontrollmechanismen reaktiver T-Zellen in der Arthritis

Die durchgeführte Studie zeigt eine zuvor unbekannte Expression von *PIR-B* in murinen T-Zellen. Diese Expression wird durch eine Störung im *TZR* Signalweg in der Peripherie und durch das komplexe inflammatorische Geschehen einer Arthritiserkrankung gefördert.

T-Zell-Antworten müssen nach einer gewünschten immunologischen Reaktion wieder abgeschaltet werden können. Dies geschieht u.a. durch die verstärkte Expression inhibitorischer Moleküle wie beispielsweise *CTLA*-4 oder *PD-1* in Effektor T-Zellen. Bei Vorliegen von Autoimmunität können diese Mechanismen gestört sein. Möglicherweise sind alternative Mechanismen vorhanden, die es zu finden gilt. Die Abschaltung/Begrenzung von initial unerwünschten autoreaktiven T-Zell-Antworten stellt eine besondere Herausforderung für das Immunsystem dar. *PIR-B* wird in murinen CD4 und CD8 T-Zellen im Verlauf einer Arthritiserkrankung hochreguliert und ist am stärksten bei Tieren mit einem verminderten Arthritisscore exprimiert (Rothe et al. 2017). Diese Tatsache lässt die Annahme zu, dass *PIR-B* die Immunantwort der T-Zellen herunterreguliert, also als ein Abschalter fungiert. Spannend ist, dass *PIR-B*$^+$ CD4 T-Zellen mehr *IFN*γ als *PIR-B* negative Zellen sezernieren und die ex vivo Analyse der IL-17 Produzenten blieb negativ. Erst eine vitro Restimulation zeigte die Kapazität der Zellen zur IL-17 Produktion in 40 Wochen alten arthritischen SKG Tieren (Rothe et al. 2017) im Kontrast zu jüngeren SKG Tieren in früheren Untersuchungen von Hirota et al. (Hirota et al. 2007). *IFN*γ hat einen sehr breiten Aktionsradius, es gilt als inflammatorischer Mediator, aber ebenfalls als Suppressor, durch die Fähigkeit, die Th17 Differenzierung in SKG Mäusen zu blockieren (Hirota et al. 2007). Infolge einer experimentell herbeigeführten Überexpression von *PIR-B* in peripheren T-Zellen aus Balb/c Tieren kommt es zu einer verminderten Th-1 Immunantwort (Imada et al. 2009). Dies unterstützt die von uns identifizierte inhibitorische Funktion des Moleküls in T-Zellen. PIR-B entfaltet ebenfalls inhibitorische Kompetenz in anderen Immunzellen (Ujike et al. 2002, Karo-Atar et al. 2013). Dies ist zusätzlich gut vereinbar mit seinem intrazellulär inhibitorisch-wirkenden Signalweiterleiter, ITIM (intracellular inhibitory motif) (Kubagawa et al. 1997).

PD-1 wurde verstärkt in T-Zellen aus dem Synovium gefunden, wodurch ein kontrolliertes Abschalten der T-Zell-Reaktionen ermöglicht scheint. In der Tat bleiben die synovialen T-Zellen aber insbesondere bei der reaktiven Arthritis in hohem Maße aktiv, die Abschaltung der T-Zell-Antwort durch PD-1 am Ort des inflammatorischen

Geschehens ist also beeinträchtigt (Raptopoulou et al. 2010). Dies zeigt auf, dass regulatorische Mechanismen vielschichtig gesteuert sind und durch das lokale Mikromilieu kontrolliert werden.

PIR-B knockout Mäuse zeigen keine offensichtlichen Veränderungen im T-Zell-Kompartiment des Immunsystems und zeigen auch keinen autoimmunen Phänotyp (Imada et al. 2009). Dies deckt sich mit unseren Daten insofern, dass *PIR-B* nicht für die T-Zell-Entwicklung wichtig ist, des Weiteren *PIR-B* nicht der Initiator einer multiparameterbedingten Autoimmunität, sondern erst infolge einer solchen induziert wird und als Regulator fungieren kann.

Kürzlich konnte durch Einführen der SKG Mutation in C57/BL6 und –BL10 Tieren eine gleiche Symptomatik mit einer gestörten T-Zell-Selektion im Thymus, einer veränderten T-Zell-Aktivierung und mit Formen der Arthritis nach Mannangabe, induziert werden (Guerard et al. 2016). Diese Tiere ermöglichen ein breiteres Spektrum der Erforschung der genauen Kostimulator- und Koinhibitor-Signalkaskaden in T-Zellen verschiedener Subtypen durch Einkreuzungen von transgenen bzw. knockout Mäusen, die weitestgehend für den C57/BL6 Background vorliegen. Darüber hinaus wären diese Tiere hinsichtlich der Untersuchung zur *PIR-B* Expression ein relevantes Modellsystem, dass unsere Daten im erweiterten Kontext validieren könnten.

Effektor T-Zellen können im Vergleich zu naiven T-Zellen TZR Signale zusätzlich zur TZR-CD3zeta-ZAP70 Kaskade über einen alternativen Signalweg, den TZR-FcRγ-Syk Weg, weitergeben (Krishnan et al. 2003). Inwieweit dieser alternative TZR Signalweg für die Expression von *PIR-B* in der Peripherie eine Rolle spielt ist unbekannt und wäre ein weiterer lohnender experimenteller Ansatzpunkt.

Zap70 Defizienz durch Mutationen im Menschen führt zu einem dem Scid Syndrom ähnlichen Phänomen, dabei sind $CD4^+$ T-Zellen meist nur gering verändert, $CD8^+$ T-Zellen stark reduziert oder fehlen in der Peripherie gänzlich (Shirkani et al. 2017). Zusätzlich zu diesem T-Zell-Entwicklungsstopp hat das Fehlen/ die eingeschränkte Funktion von ZAP70 Auswirkungen auf die Stimulierbarkeit der peripheren $CD4^+$ T-Zellen (Kaur et al. 2014). Dies geht klinisch einher mit schwer kontrollierbaren bakteriellen, viralen und fungalen Infektionen. Zusätzlich findet sich bei Menschen mit einem ZAP-70 Defekt ein vermindertes peripheres T-Zell-Repertoire (Roifman et al. 2010), ähnlich wie bei Patienten/Patientinnen mit einer rheumatoiden Arthritis (Wagner et al. 1998) und anderen T-zellassoziierten Autoimmunkrankheiten.

Der Befund einer höheren Expression von *PIR-B* in T-Zellen in Abhängigkeit vom Mikrobiom bietet wegweisende neu Ansatzpunkte. Unveröffentlichte Daten demonstrieren eine verstärkte Expression von *PIR-B* nach einer CMV Infektion von SKG Tieren im Vergleich zu altersidentischen und bezüglich der Arthritis klinisch vergleichbaren, nicht infizierten Tieren.
PIR-B scheint daher sowohl infolge einer T-zellulären Immunantwort gegen Pathogene, als auch infolge einer Auseinandersetzung des Immunsystem mit Autoimmunität exprimiert zu werden. Dies sollte in anderen experimentellen Mausmodellen, beispielsweise der experimentellen autoimmunen Enzephalomyelitis und durch Infektion von CMV bzw. anderer Pathogene in Wildtyp-Mausstämmen, evaluiert werden.

3.2. Die Bedeutung von Kostimulatoren und Koinhibitoren für die T-Zell-Homöostase und die antitumorale T-zelluläre Immunabwehr

3.2.1. T-Zell-Aktivierung durch B7-H3 auf Hautzellen

Die Haut zählt zu den wichtigsten Organen der primären Abwehrlinie gegen Erreger aus der Umwelt. Sie arbeitet synergistisch mit anderen Abwehrlinien, beispielsweise den T-Zellen der adaptiven Immunität zusammen. Die in dieser Forschungsarbeit gewonnenen Ergebnisse der *B7-H3* Expression auf Keratinozyten der Epidermis, die die hömostatische T-Zellproliferation steuern, tragen somit wesentlich zum Verständnis der Zusammenarbeit dieser Systeme bei.

In Erwachsenen wird die Aufrechterhaltung des T-Zell-Pools durch verschiedenste Organsysteme erfüllt, nur ca. 2,2% aller Lymphozyten zirkulieren im Blut. Die überwiegende Zahl befindet sich in Lymphknoten, gefolgt von anderen primären und sekundären Lymphorganen, wobei ebenfalls nur ca. 2% in anderen Organsystemen verweilen (Ganusov and De Boer 2007). Interessanterweise beherbergt die Haut eines Menschen ca. 2x mehr T-Zellen als sich in der Zirkulation befinden, was die T-zellbiologischen Vorgänge in der Haut um so relevanter macht (Clark 2010).

Die Ergebnisse der *B7-H3* Studie zeigen eine gleichartig hohe Expression von *B7-H3* auf Transkript- und Proteinebene in den Keratinozyten der oberen Hautschicht. Das unterscheidet dieses gesunde Gewebe von anderen Geweben, bei denen vielfach eine posttranskriptionale Kontrolle die Proteinexpression von *B7-H3* trotz hoher Transkriptmengen unterbindet (Chapoval et al. 2001).

Im Gegensatz zu *B7-H1* und *B7-H4* ist die Affilierung von *B7-H3* als Kostimulator bzw. Koinhibitor nicht festgelegt, sondern vielmehr zellkontext- und rezeptorabhängig (Chapoval et al. 2001, Suh et al. 2003).

In der hier dokumentierten Studie konnte *B7-H3* als Kostimulator der T-Zell-Antwort identifiziert werden. Weitere eigene unveröffentlichte Daten im Nierenzellkarzinom zeigen einen signifikant geringeren Primärtumorumfang *in situ* bei hoher tumoraler *B7-H3* Expression, einhergehend mit einem erhöhten CD8 T-Zell-Infiltrat und erniedrigtem CD4 T-Zell-Infiltrat. Dies spricht ebenfalls für einen kostimulierenden Effekt einer CD8 T-Zell-Antwort durch *B7-H3* auf Nierenkarzinomzelllinien. Andere Arbeiten zur Bedeutung von *B7-H3* bei Tumoren reihen sich ein in die Divergenz der Funktion des Moleküls. Es wurde eine tumorunterstützende Rolle für zervikale und Lungen-Tumore (Mao et al. 2015, Li et al. 2017) und eine tumorinhibierende Wirkung im Pankreaskarzinom (Loos et al. 2009) gefunden.

Wichtig zu betrachten ist der Fakt, dass es oft zu einer koordinierten parallelen oder zeitlich begrenzten, sich verändernden Expression multipler Liganden und Rezeptoren der *B7* Familie, wie auch der Kostimulatoren/Koinhibitoren anderer Familien, z.B der *TNF/TNF*-Rezeptor Familie, auf den beteiligten Zellen kommen kann. Insofern sind Ergebnisse aus einer isolierten experimentellen Fokussierung auf eines der Ligand/Rezeptorpaare immer kritisch zu betrachten und decken nicht die ganze Bandbreite zellbiologischer Prozesse *in vivo* ab.

Ein weiterer Forschungsansatz von hoher Relevanz wäre die Untersuchung der funktionellen Konsequenz eines über *B7-H3* vermittelten Signals für die Keratinozyten. Dies geht auf Forschungsarbeiten zurück die zeigen konnten, dass *B7-H3* selbst als Rezeptor fungieren kann und in anderen Geweben die Zelldifferenzierung der *B7-H3* tragenden Zelle steuert (Xu et al. 2011).

3.2.2. T-Zell-Kontrolle durch Koinhibitorexpression auf Tumorzellen

Das Tumormikromilieu wird neben den Tumorzellen durch verschiedenste Zelltypen wie Immunzellen, Fibroblasten, Adipozyten, Perizyten, Endothelzellen und Stammzellen, darüber hinaus aus nicht-zellulären Bestandteilen der extrazellulären Matrix gebildet. Metabolische Produkte und zelluläre Botenstoffe aller vorhandenen Zelltypen bestimmen das Geschehen (Reina-Campos et al. 2017). Zytokine und Chemokine sind essentielle zelluläre Botenstoffe der Immunzellen, aber auch der

Gewebszellen verschiedener Organe und der Gefäßzellen, die das Tumorgewebe solider Tumoren vaskularisieren. Die durchgeführte Studie zur Regulation der B7-H Moleküle durch Zytokine spiegelt in Teilen experimentell diese komplexe Situation verschiedener Botenstoffe wieder. Die konstitutive Analyse der *B7-H* Expression in Nierenkarzinomzelllinien bestätigte publizierte Expressionsmuster von *B7-H1* (Thompson et al. 2006), B7-H3 (Crispen et al. 2008) und *B7-H4* (Krambeck et al. 2006). *B7-H2* (*ICOS-L*) Expression und Regulation hingegen wurde durch unsere Forschungsarbeit erstmals beschrieben. Fortführende Untersuchungen zur Rolle von *B7-H2* für das Nierenzellkarzinom könnten sich sinnvoll anschließen, denn $CD4^+$ T-Zellen, die Hauptträger des ICOS Rezeptors, wurden bereits sehr früh als wichtige Kontributoren der anti-tumoralen Immunantwort identifiziert (Pardoll and Topalian 1998).

Die Ergebnisse unserer Studie des synergistischen Effektes anti-inflammatorischer und pro-inflammatorischer Zytokine auf die *B7-H1* Expression sind wichtig für das Verständnis der patienten-/patientinnenspezifischen Expressionsdaten von *B7-H1* auf Tumorzellen, die sich durch das unterschiedliche Mikromilieu bestimmen können. Begründet durch die hohe Variabilität des Immunsystems und der Tumore der Patienten/Patientinnen wird moderne Krebstherapie zunehmend zur individualisierten Medizin (Quandt et al. 2017). Die untersuchten Zytokine werden im Wesentlichen durch das Immunzellinfiltrat im Tumor sezerniert. Zusätzlich zu den T-Zellen und myeloiden Zellen sind auch B-Zellen vor Ort. B-Zellen können über verschiedene Mechanismen, u.a. durch Zytokinsezernierung von z.B. *IL-10* oder *IL-4* (Harris et al. 2000), aktiv das Geschehen im Tumormikromilieu beeinflussen (Cai et al. 2016, Chiaruttini et al. 2017). B-Zellen sind im Tumormikromilieu hochorganisiert, sie befinden sich in tertiären lymphoiden Strukturen (*TLS*) (Dieu-Nosjean et al. 2016), die Ähnlichkeiten mit der Organisation in Lymphknoten, den sekundären lymphatischen Geweben, aufweisen.

Neben der Regulation durch Zytokine gibt es andere Signalwege, die eine aberrante *B7-H1* Expression in Tumoren bewirken. Hier sind Mutationen in der 3'UTR des Gens (Kataoka et al. 2016), und Veränderungen in *STAT3* und *AKT* Signalwegen zu nennen (Parsa et al. 2007, Marzec et al. 2008). *PD-1* und *B7-H1* (*PD-L1*) sind mittlerweile Zielstrukturen moderner Immuntherapien im Nierenzellkarzinom (Atkins et al. 2017, Mazza et al. 2017). Trotz dieser erfreulichen Entwicklung neuer Therapieoptionen gibt es Patienten/Patientinnen, die von diesen Therapieansätzen

nicht profitieren. Der zunächst naheliegendste Grund für ein Nicht-Ansprechen auf die anti-PD-L1 Therapie, eine unterschiedliche Expression von *B7-H1* (*PD-L1*) auf den Tumorzellen, hat sich nur teilweise bestätigt. Wichtig für ein Therapieansprechen scheint eine PD-L1 Expression auf verschiedenen Immunzellen im Tumormikromilieu zu sein (Herbst et al. 2014, Quandt et al. 2017). In präklinischen Modellen konnte ein Ansprechen der anti-*PD-L1* und anti-*PD-1* Therapie trotz fehlender tumoraler *B7-H1* (PD-L1) Expression aufgezeigt werden (Kleinovink et al. 2017). Mit Nachdruck wird in der sogenannten „liquid biopsy", dem Blut, nach Markern/Markerprofilen, die ein Ansprechens bzw. Nicht-Ansprechen auf diese Immuntherapien vorhersagen gesucht. Es wird ein bestimmtes Immunprofil bzw. ein zu Beginn der Therapie bestehender kostimulatorischer/koinhibitorischer Rezeptorstatus auf den Immunzellen vermutet.

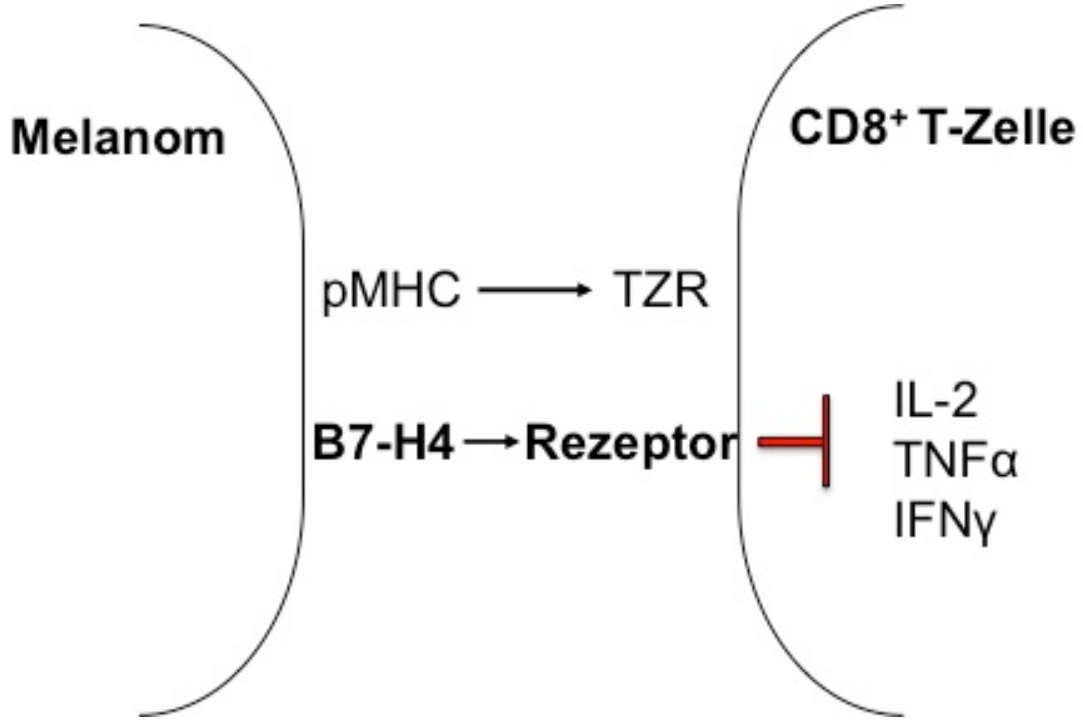

Abb.6 B7-H4 als neue Zielstruktur im malignen Melanom B7-H4 tragende Tumorzellen inhibieren die Zytokinsekretion und damit eine essentielle Effektorfunktion tumorspezifischer $CD8^+$ T-Zellen.

Die Resultate unserer Studie zu *B7-H4* im Melanom zeigen die Bedeutung des Moleküls für die Pathogenese dieser Tumorerkrankung auf. *B7-H4* auf Melanomzellen dient möglicherweise, ähnlich wie *B7-H1,* als Tumorescape-Strategie,

denn die antitumorale Immunantwort wird über dieses Ligand-Rezeptorpaar unterdrückt (Abb. 6).
Die Ergebnisse der Untersuchungen zu *B7-H4* im Melanom wurden vielfach zitiert und fügen sich in die Reihe verschiedener Tumorentitäten, für die *B7-H4* als prognostisch relevante Zielstruktur identifiziert wurde, ein (Podojil and Miller 2017).

Interessanterweise wird *B7-H4* nicht nur auf den Tumorzellen, sondern ebenfalls auf Immunzellen des Tumormikromilieus, z.B. den Tumor-assoziierten Makrophagen (TAM), überexprimiert, wodurch es zu einer Inhibition der $CD8^+$ T-Zellen kommt (Kryczek et al. 2006). Wir fanden in unserer Studie signifikant erhöhte Zahlen an TAMs in den Patienten/Patientinnen mit kürzerem Überleben. Das Immunzellinfiltrat im hier untersuchten Patienten/-Patientinnenmaterial bestand vor allem aus Makrophagen und $CD8^+$ T-Zellen und zeigte ebenfalls eine *B7-H4* Überexpression, was trotz fehlender Kofärbung zur Identifizierung des Immunzelltyps den Schluss nahe legt, dass auch im Melanom inhibitorisch wirkende Makrophagen an der Pathogenese beteiligt sind. Der Rezeptor für *B7-H4* auf T-Zellen ist immer noch nicht identifiziert (Podojil and Miller 2017). Diese Identifikation des Rezeptors ist ein wichtiger experimenteller Forschungsansatz, denn so könnte ähnlich wie für *PD-1* (Rezeptor von B7-H1, (Guo et al. 2017)) der Rezeptor für *B7-H4* selbst direkt Angriffspunkt für neue therapeutische Ansätze sein.

Zusammenfassung

Autoimmunkrankheiten wie die rheumatoide Arthritis und maligne Neoplasien verursachen erhebliche Kosten und Arbeitsausfall für die Gesellschaft und persönliches Leid für den betroffenen Patienten/Patientinnen.

Das Durchleben von Infektionen, das Auftreten von Autoimmunkrankheiten und die Bildung maligner Neoplasien sind scheinbar getrennte Ereignisse. Die Ergebnisse dieser Arbeit und weltweit durchgeführter Analysen decken eine enge immunologische Verzahnung dieser Ereignisse auf, die deutlich machen, dass sich ein „erinnerndes“ Immunsystem diesen Aufgaben in Abhängigkeit einer individuell verschiedenen Patienten-/Patientinnenhistorie widmet und ursächlich zum Auftreten dieser Erkrankungen beiträgt.

Chronische Infektionen wie CMV verändern den zellulären Immunstatus maßgeblich und sollten daher klinisch Berücksichtigung finden. Es wird zunehmend davon ausgegangen, dass der Verlauf und die Therapieoptionen dieser Erkrankungen vom immunologischen Profil der Patienten/Patientinnen abhängen.

Trotz deutlicher Fortschritte in der Behandlung, wie beispielsweise durch den Einsatz der *TNFα* Therapie bei der rheumatoiden Arthritis und den Einsatz der neuen Immuntherapien zur Behandlung von malignen Neoplasien, bleiben viele Patienten/Patientinnen therapeutisch unterversorgt.
Daher ist ein tieferes Verständnis der immunologischen Vorgänge, die ursächlich krankheitsrelevant bzw. im Verlauf der Erkrankung zur Bekämpfung selbiger beitragen können, notwendig, um weitere Therapieoptionen implementieren zu können.
T-Zellen als Vertreter der adaptiven Immunität spiegeln pathologische Vorgänge wieder und geben dabei Rückschlussmöglichkeiten für den Progress und bilden selbst therapeutische Zielstrukturen zur Behandlung.

Das Verständnis allgemeingültiger sowohl als auch krankheitsspezifischer T-zellbiologischer Wirkweisen eröffnet neue Denkstrukturen und Forschungsansätze.

Die in den hier vorgestellten kliniknahen grundlagenwissenschaftlichen Forschungsarbeiten dokumentierten Ergebnisse zur Rolle von $CD4^+CD28^-$, $CD8^+$*LIR-1*$^+$, $CD4^+$/*PIR-B*$^+$ und $CD4^+CD8^+$ doppelt positiven T-Zellen im Kontext mit rheumatischen Erkrankungen mit vorgeschalteter persistierender CMV Infektion tragen zur weiteren Wissensvertiefung der T-zellimmunologischen Vorgänge bei.
Die durchgeführten tumorimmunologischen Untersuchungen mit Fokus auf die Familie kostimulatorischer Moleküle der *B7*-Familie im translationalen Forschungsgebiet solider Tumore führten zur Aufdeckung bisher unbekannter T-zellulärer Mechanismen, deren Kenntnis in zukünftige Therapieentwicklungen und die Erweiterung des Einsatzes bestehender Therapieoptionen einfließen sollten.

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Thesen

1. T-Zellen sind hochspezialisierte Zellen des adaptiven Immunsystems. Sie kooperieren mit dem angeborenen Immunsystem und bilden einen fundamentalen Teil des Abwehrsystems höherer Organismen. T-Zellen verschiedener Subtypen greifen in die Pathogenese sowohl von Autoimmunkrankheiten als auch malignen Neoplasien ein. Krankheitsübergreifende Mechanismen der T-Zell-Aktivierung, -Effektorfunktion und -Inhibition konnten in der vorliegenden Arbeit identifiziert werden.

2. Virusinfektionen können Auslöser oder Verstärker diverser Autoimmunkrankheiten als auch Krebserkrankungen sein und sind daher Gegenstand intensiver Forschung und bieten Ansatzpunkte für neue Therapien.

3. Immuntherapien sind eine vielversprechende neue Therapiestrategie gegen Krebserkrankungen. Insbesondere Moleküle die T-Zell-Antworten steuern, werden adressiert. Dabei spielt sowohl der patienten-/patientinnenspezifische Zustand des Immunsystems als auch die molekulare Signatur des individuellen Tumors eine bedeutende Rolle.

4. Eine persistierende CMV Infektion führt zu einem schwereren Krankheitsverlauf bei Patienten/Patientinnen mit rheumatoider Arthritis (RA). Sowohl $CD4^+$ als auch $CD8^+$ T-Zellen weisen CMV-spezifische phänotypische und funktionelle Veränderungen auf, die mit klinischen Daten korreliert werden konnten. *LIR-1* wird verstärkt auf $CD8^+$ T-Zellen in CMV positiven RA-Patienten/-Patientinnen exprimiert. Die Kontrolle der erhöhten zytolytischen Aktivität der $CD8^+$ T-Zellen durch den biologischen Liganden *HLA-G in vitro*, belegt die Funktionalität von LIR-1 in RA-Patienten/-Patientinnen. Das mit CMV und RA konfrontierte T-zelluläre Immunsystem kann sich *in vivo* nicht mehr ausreichend über *LIR-1* kontrollieren. Überschießende Zytokine und höhere zytolytische T-Zell-Reaktivitäten tragen bei CMV positiven RA-Patienten/-Patientinnen zum schwereren Krankheitsverlauf bei.

5. Periphere doppelt positive ($CD4^+CD8^+$) T-Zellen (DP T-Zellen) konnten erstmals als signifikant veränderte T-Zell-Subpopulation bei RA-Patienten/-Patientinnen identifiziert werden. Die Zellen wiesen einen von den singulär CD4 bzw. CD8

Korezeptor-tragenden T-Zellen distinkten Phänotyp auf. DP T-Zellen bei RA-Patienten/-Patientinnen sind polyfunktionell und mit einem signifikant höheren Effektorpotential als DP T-Zellen gesunder Probanden ausgestattet. Diese T-Zell-Subpopulation ist eng mit dem Auftreten von für die RA typischen Autoantikörpern assoziiert. In CMV positiven RA-Patienten/-Patientinnen tragen sie zu der erhöhten Pathogenität CMV-spezifischer T-Zellen bei.

6. Das *LIR-1* orthologe *PIR-B* wurde erstmals in murinen T-Zellen detektiert. Die Expression wird maßgeblich durch ein inflammatorisches Milieu gefördert, in dem es zur Aktivierung der T-Zellen kommt. Ex vivo Analysen von T-Zellen aus Tieren mit einer Spontanarthritis (SKG Modell) wiesen hohe *PIR-B* Expressionslevel auf. *PIR-B* positive T-Zellen produzieren hauptsächlich regulatorisch wirkendes *IFNγ*, nicht *IL-17*. Die funktionelle Ausgestaltung *PIR-B* positiver T-Zellen geht sehr gut mit dem klinischen Befund einher - höhere *PIR-B* Expressionslevel korrelieren mit einem milderen Arthritisverlauf.

7. Die Kombination anti-inflammatorischer und inflammatorischer Zytokine führte selektiv zur synergistischen Induktion von *B7-H1* (*PD-L1*) auf Nierenkarzinomzelllinien. Andere *B7* Familienmitglieder blieben in ihrer Expression von einer kombinatorischen Zytokingabe unbeeinflusst. Beteiligte Signalkaskaden schließen *STAT6* und *NFκB* ein. Die zytokin-induzierte Regulation von *B7-H1* ist mit immunmodulatorischen Konsequenzen für $CD8^+$ T-Zellen verbunden. Dieser Befund ist wichtig für moderne immuntherapeutische Krebsbehandlung, bei der Signalwege über *B7-H1* unterbunden werden, wodurch das T-zelluläre Immunsystem Aktionsspielraum zurückgewinnt.

8. Der Kostimulator *B7-H3* wurde erstmals auf humanen gesunden Keratinozyten *in situ* und *in vitro* an Primärzellen und Zelllinien auf Transkript- und Proteinebene nachgewiesen. Funktionell konnte dem Molekül eine unterstützende Rolle für die Proliferation und Zytokinsekretion $CD8^+$ positiver T-Zellen zugeordnet werden. Die konstitutiv hohe Expression von *B7-H3* in der gesunden Haut lässt den Schluss einer die T-Zell-Homöostase fördernden Wirkung des Kostimulators zu.

9. *B7-H4* konnte als neue Zielstruktur im malignen Melanom identifiziert werden. Primärtumore und Metastasen zeigen signifikant erhöhte Expression von *B7-H4* gegenüber gesunder Haut. Hohe Expression von *B7-H4* auf Tumorzellen *in situ* konnte mit einem kürzeren Überleben der Patienten/Patientinnen assoziiert werden. *B7-H4* auf Melanomzelllinien inhibiert die anti-tumorale Immunantwort durch eine Blockade der Zytokinproduktion Antigen-spezifischer CD8 Effektor-T-Zellen.

10. Die Resultate der vorliegenden Arbeit unterstreichen die bedeutende Rolle verschiedener T-Zell-Populationen für das Auftreten und die Pathogenese von Autoimmunkrankheiten und Krebserkrankungen. Es wurden neue T-zellspezifische Kontrollmechanismen im Kontext beider Erkrankungsbilder identifiziert, die therapeutische Ansatzpunkte eröffnen.

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Association of Anticytomegalovirus Seropositivity With More Severe Joint Destruction and More Frequent Joint Surgery in Rheumatoid Arthritis

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Objective. **Expansion of autoreactive CD4+ $CD28^{null}$ T cells is associated with extraarticular disease manifestations, including rheumatoid vasculitis, and it has recently been demonstrated that expansion of these T cells is associated with anticytomegalovirus (anti-CMV) seropositivity. This study was undertaken to investigate a possible link between latent CMV infection and rheumatoid arthritis (RA).**

Methods. **In a retrospective analysis, anti-CMV antibodies and clinical, serologic, and radiologic parameters of joint destruction were examined in 202 RA patients and 272 healthy controls. In addition, frequencies of CD4+$CD28^{null}$ T cells; concentrations of the cytokines monocyte chemotactic protein 1 (MCP-1), interferon-α (IFNα), and IFN-inducible protein 10; and anti-CMV–specific T cell responses were analyzed in RA patients.**

Results. **Overall, no significant difference in the frequency of anti-CMV seropositivity between RA patients and healthy controls was observed. Among individuals older than age 55 years, however, anti-CMV IgG antibodies were significantly more frequent in RA patients than controls (65.3% and 54.7%, respectively; $P = 0.05$). Anti-CMV seropositivity in RA patients was associated with an increased frequency of CD4+$CD28^{null}$ T cells and increased serum concentrations of MCP-1. The frequency of anti-CMV–specific CD4+ T cells producing IFNγ was increased in RA patients compared to controls. Most importantly, anti-CMV–seropositive RA patients showed radiographic evidence of more advanced joint destruction and had increased frequencies of joint-related surgical procedures, indicating more severe joint disease.**

Conclusion. **Our findings indicate that latent CMV infection aggravates the clinical course of RA and is associated with increased frequencies of CD4+ $CD28^{null}$ T cells and of CMV-specific IFNγ-secreting CD4+ T cells.**

In rheumatoid arthritis (RA), disturbances in the T cell pool and associations with polymorphisms in T cell genes strongly implicate CD4+ T cells in disease pathogenesis. Phenotypically, the peripheral T cell pool in RA patients is characterized by large and stable clonal expansions, and by an increased frequency of CD4+ T cells that have lost expression of the ubiquitously expressed costimulatory molecule CD28 but have gained expression of killer cell immunoglobulin-like receptor (KIR) (1). Simultaneously, the global CD4+ T cell pool in RA patients has an increased replicative history compared to that in age-matched controls, which leads to premature erosion of telomere fragments in both naive and memory CD4+ T cells (2). It can be estimated that the immune system of RA patients has grown >10 years older than that of age-matched controls in several aging parameters, due to premature and accelerated immunosenescence. Of clinical relevance is the association of clonal expansions and increased frequencies of CD4+$CD28^{null}$ T cells with extraarticular manifestations of the disease (3) and, in particular, with rheumatoid vasculitis (4).

Cytomegalovirus (CMV) infection causes clinically severe infections in immunocompromised patients

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and exerts a profound influence on the peripheral, circulating T cell pool in healthy individuals without apparent clinical disease. The involvement of a large fraction of the peripheral T cell pool in the CMV-induced immune response becomes evident in a massive proliferative expansion, which has been called memory inflation and which can be observed in both CD4+ and CD8+ T cells (5,6). As a result, anti-CMV seropositivity is associated with accelerated immunosenescence of CD4+ and CD8+ T cells in otherwise healthy individuals (7) and shows several parallels with the premature aging of the immune system that is a hallmark of RA. In particular, the emergence of CD4+CD28^{null} T cell clones following CMV infection (8), which is also accompanied by a gain in the expression of KIR (9), has indicated a possible link between RA and CMV infection.

In patients with RA, a clear association of anti-CMV seropositivity with the expansion of CD4+CD28^{null} T cells has previously been established (10,11), but no systematic analysis of its impact on disease severity and extraarticular manifestations has been published. Given the strong influence of CMV infection on the emergence of pathologic CD4+CD28^{null} KIR-expressing T cells, which are frequently associated with vasculitis, we investigated a possible link between CMV infection, disease susceptibility, and the clinical course of RA.

PATIENTS AND METHODS

Patient population. Patients with RA (n = 202) were recruited from the rheumatology unit of the University of Leipzig and enrolled in this study analyzing serologic CMV status. The study was approved by the local ethics committee, and informed consent was obtained from all patients. As a control group, 272 donors, who were selected for an age range equivalent to that in the patient cohort, were recruited from the local blood bank. Flow cytometric data on CD28 expression on CD4+ T cells were available for 139 RA patients. For the analysis of CMV-induced T cell proliferation, 17 patients and 11 controls were included, and for the analysis of interferon-γ (IFNγ) production, 7 patients and 7 controls were included.

Collection of clinical data. All study patients were receiving clinical care at the rheumatology unit, and a thorough chart review was performed at the time of study enrollment. Based on the report of the last available hand and feet radiographs, the documented disease stage according to the Steinbrocker criteria was determined in all patients (12,13). In addition, results of testing for anti–cyclic citrullinated peptide (anti-CCP) antibodies, rheumatoid factor (RF), and antinuclear antibody; results of serologic testing for anti-CMV seropositivity; and current C-reactive protein levels were available for all patients and were documented for the study. All previously prescribed disease-modifying antirheumatic drugs (DMARDs), the duration of treatment and the cause of discontinuation, as well as the history of joint-related surgical procedures were recorded. Surgical procedures performed in the patients included synovectomy, tenosynovectomy, osteotomy for the correction of bone malalignment, arthrodesis, and joint replacement surgery; the numbers of times these procedures were performed were documented.

Flow cytometric analysis and measurement of serum cytokine levels by enzyme-linked immunosorbent assay (ELISA). For the quantification of CD4+CD28^{null} T cells, flow cytometry was performed as previously described (3). For the determination of human monocyte chemotactic protein 1 (MCP-1) concentrations, a commercially available kit consisting of a capture antibody and a detection antibody (DuoSet ELISA Development kit, catalog no. DY279; R&D Systems) (detection limit 7.8 pg/ml) was used to establish the ELISA according to the manufacturer's instructions. Similarly, a DuoSet ELISA Development kit (catalog no. DY266; R&D Systems) (detection limit 15.6 pg/ml) was used to establish an ELISA for human IFN-inducible protein 10 (IP-10). Serum concentrations of IFNα were determined by an ELISA on the basis of a matched antibody pair (catalog no. BMS216MST; Bender MedSystems) (detection limit 15.6 pg/ml).

Analysis of CMV-specific proliferation and of IFNγ production by T cells. Peripheral blood mononuclear cells (PBMCs) from anti-CMV-seropositive RA patients and healthy controls were separated by density-gradient centrifugation. Samples from RA patients who were anti-CCP positive and age-matched healthy controls were included in the analysis. For quantification of cell division, a fluorescence-based proliferation assay was performed as previously described (14), by labeling with 5,6-carboxyfluorescein succinimidyl ester (CFSE; Molecular Probes). For the proliferation analysis, PBMCs (1×10^6) were cultured for 7 days in the presence or absence of 1 μg/ml CMVpp65 peptide mixture or control peptide (15 mers, 11 amino acid overlap; Jerini Peptide Technologies) or 3 μg/ml CMV lysate or control lysate (Microbrix Biosystems) in 24-well plates in X-Vivo 15 (Lonza) containing 2 m*M* L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. For the analysis of IFNγ secretion, PBMCs (2×10^6) were cultured for 4 hours under the same conditions as described for the proliferation analysis. The IFNγ secretion assay was performed according to the recommendations of the manufacturer (Miltenyi Biotec). In all experiments using this assay, stimulation of T cells using CytoStim (Miltenyi Biotec) was included as a positive control and showed similar frequencies of IFNγ producers among RA and healthy donor samples.

The following antibodies were used for counterstaining T cells in fluorescent conjugates of phycoerythrin or allophycocyanin: anti-CD4 (clone M-T466), anti-CD8 (clone BW135/80), anti-CD3 (clone BW264/56), and anti-CD71 (clone AC102) (all from Miltenyi Biotec). Dead cells were removed from the analyses by the exclusion of propidium iodide–positive cells.

Statistical analysis. Differences in medians or means between groups were analyzed by Student's *t*-test or the Mann-Whitney rank sum test as appropriate. Correlations were evaluated using Pearson's product-moment correlation or Spearman's rank correlation coefficient. Logistic regression analysis was performed by stepwise removal of independent variables using the likelihood ratio test as the criterion. Odds ratios (ORs) in multiple logistic regression analysis for inde-

pendent variables were estimated by OR = eβ, where β is the regression coefficient, as previously described (15).

RESULTS

Analysis of anti-CMV positivity in RA patients and healthy controls. Sera from 202 patients with RA and from 272 healthy controls were analyzed for the presence of anti-CMV IgG antibodies. The clinical characteristics of the patients are shown in Table 1. The frequency of anti-CMV seropositivity was 56.93% in RA patients and 54.04% in healthy controls, with no significant difference between the 2 populations ($P = 0.595$). Similarly, no difference in the anti-CMV IgG concentration between RA patients and controls was detectable.

In initial bivariate comparisons, anti-CMV–positive patients were found to be significantly older than anti-CMV–negative patients (median age 64.2 years and 58.5 years, respectively; $P < 0.001$). Consistent with their older age, anti-CMV–positive patients also had a longer disease duration between the onset of symptoms and the time of analysis, with a median of 13.5 years (interquartile range [IQR] 6–23 years) in anti-CMV–positive patients versus a median of 8 years (IQR 3–17 years) in anti-CMV–negative patients ($P = 0.001$). A trend toward an earlier disease onset in anti-CMV–positive patients did not reach statistical significance (mean age at disease onset 44.5 years in anti-CMV–seropositive patients versus 46.8 years in anti-CMV–seronegative patients; $P = 0.25$).

The observed influence of age on the frequency of latent CMV infection prompted us to perform subgroup analyses in order to investigate the rate of CMV seropositivity in different age groups. The percentage of RA patients older than age 55 years who were anti-CMV positive was found to be significantly higher than that of healthy controls of the same age (65.3% and 54.7%, respectively; $P = 0.05$).

Serologic reactivity with CMV has previously been shown to be associated with the expansion of a CD4+CD28^{null} T cell compartment both in healthy individuals and in RA patients (11,16). For our study cohort, flow cytometric data were available for 87 anti-CMV–negative and 115 anti-CMV–positive RA patients. As previously shown, anti-CMV reactivity was associated with increased frequencies of CD4+CD28^{null} T cells (median 8.15% [IQR 1.582–20.392%] in anti-CMV–seropositive patients versus 0.37% [IQR 0.16–0.89%] in anti-CMV–seronegative patients; $P < 0.0001$). In fact, anti-CMV seropositivity appeared to be almost a

Table 1. Characteristics of the RA patients*

	All RA patients (n = 202)	Anti-CMV–negative RA patients (n = 87)	Anti-CMV–positive RA patients (n = 115)
Sex, no. male/no. female	46/156	20/67	29/86
Age, years	62.3 (51–68)	58 (45–65)	64 (57–70)
Disease duration before study entry, years	12 (5–21)	8 (3–17)	13 (6–23)
IgM-RF positive, no. (%)	180 (89.1)	78 (89.6)	102 (88.7)
IgM-RF titer	203 (68–565)	218 (68–520)	193 (68–593)
IgA-RF positive, no. (%)†	137 (81.5)	59 (80.8)	78 (82.1)
IgA-RF titer†	76 (27–188)	62 (24–191)	93 (27–188)
Anti-CCP positive, no. (%)†	157 (81.7)	65 (81.9)	108 (84.2)
Anti-CCP titer†	429 (80–1,600)	306 (46–1,600)	474 (100–1,600)
Steinbrocker radiographic stage	3 (2–4)	3 (2–4)	3 (2–4)
No. of synovectomies	1 (0–3.5)	0 (0–2)	1.5 (0–5)
Total no. of joint-related surgical procedures‡	2 (0–6)	1 (0–5)	3.5 (1–8)
No. of conventional DMARDs§	2 (1–4)	2 (1–4)	3 (1–4)
TNFα inhibitor treatment, no. (%)	39 (19.3)	18 (20.7)	21 (18.3)
Shared epitope positive, no. (%)¶	90 (71.4)	34 (73.9)	56 (71.8)
DR4 shared epitope positive, no. (%)¶	63 (50)	22 (46.8)	41 (51.9)
C-reactive protein, mg/liter¶	17 (8–36)	15 (6–38)	19 (9–36)
No. of swollen joints¶	6 (2–12)	6 (2–13)	5 (2–12)

* Except where indicated otherwise, values are the median (interquartile range). RA = rheumatoid arthritis; IgM-RF = IgM rheumatoid factor; anti-CCP = anti–cyclic citrullinated peptide; TNFα = tumor necrosis factor α.
† Data were not available for all patients.
‡ Data on surgical procedures were available for 78 anticytomegalovirus (anti-CMV)–negative and 102 anti-CMV–positive patients.
§ Number of successive treatment attempts with different disease-modifying antirheumatic drugs (DMARDs).
¶ Data were available for 126 patients.

prerequisite for the expansion, since only 3 patients in the anti-CMV–negative group had >5% CD4+CD28^{null} T cells in their peripheral blood (Figure 1A).

Association of CMV seropositivity in RA patients with increased frequencies of CMV-specific IFNγ-secreting CD4+ T cells and increased serum levels of MCP-1. Previous studies have shown that the peripheral CD4+ T cell pool in healthy individuals contains a large fraction of CMV antigen–specific T cells, which are constantly proliferating toward replicative exhaustion, and which are preferentially CD4+CD28^{null} (17). In order to test the proliferative response of CD4+ T cells from RA patients and controls toward pp65, in vitro proliferation assays of T lymphocytes were performed in cultures of PBMCs from anti-CMV–seropositive RA patients and healthy controls in the presence of either a peptide mixture from the CMV antigen pp65 or a control peptide (Figure 1B). Antigen-specific proliferation occurred in both the CD4+ (Figure 1C) and CD8+ (Figure 1D) T cell compartments, while proliferation in the control cultures was significantly lower. The frequency of proliferating CD8+ T cells was higher than that of CD4+ T cells. No significant difference between RA patients and healthy controls in the frequency of proliferated cells was detectable. Similar results were obtained when total CMV lysate was used as antigen in the cultures (Figure 1E), except that CD8 T cell responses were lower than CD4 T cell responses.

Parallel to the analysis of proliferation, expression of CD71 as a cell surface activation marker on CD4+ T cells was determined. Again, a significantly higher percentage of CD71+CD4+ T cells was found in the cultures with pp65 peptide than in those with control peptide, but no significant difference between

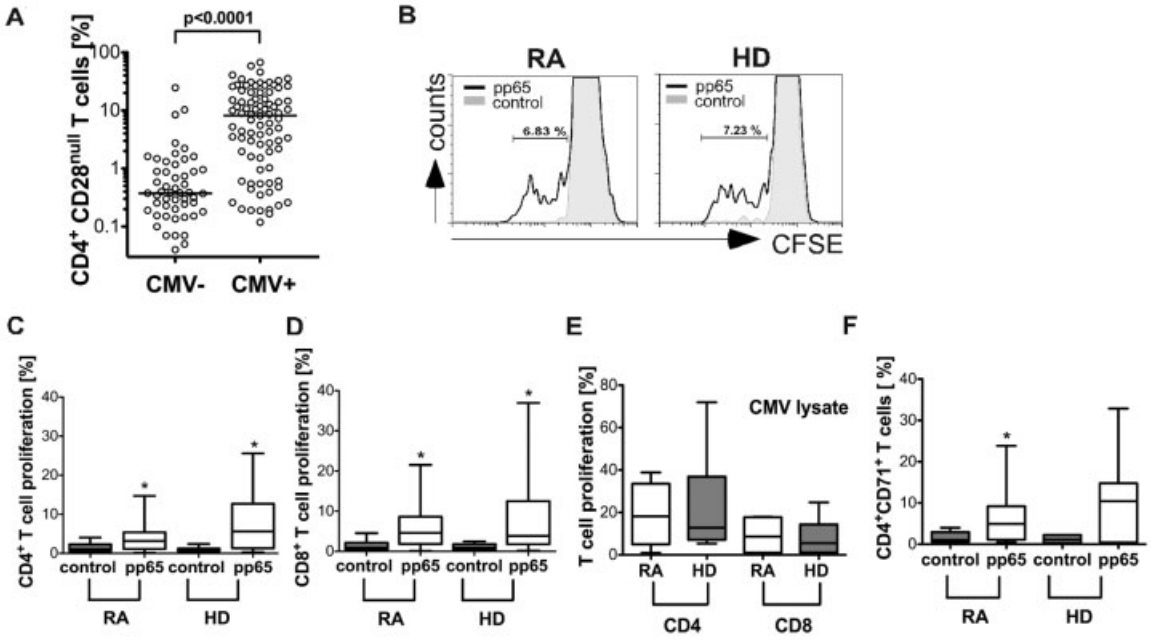

Figure 1. Association of cytomegalovirus (CMV) seropositivity with increased frequencies of CD4+CD28^{null} T cells in patients with rheumatoid arthritis (RA), but not with increased CMV-specific proliferation of CD4+ T cells. **A,** Frequency of CD4+CD28^{null} T cells as a percent of total CD4+ T cells in anti-CMV–negative (n = 54) and anti-CMV–positive (n = 85) RA patients. Circles represent individual patients; horizontal lines indicate the median. **B–F,** Peripheral blood mononuclear cells (PBMCs) from RA patients and from healthy donors (HD) were 5,6-carboxyfluorescein succinimidyl ester (CFSE)–labeled, incubated with either pp65 peptide mixture, CMV lysate, control peptide, or control lysate for 7 days, and analyzed for CFSE dilution by flow cytometry. **B,** Representative CFSE dilutions gated on CD3+CD4+ live (by propidium iodide exclusion) T cells in RA patients and healthy donors for control peptide or pp65 peptide mixture. **C** and **D,** Percentage of proliferated CD4+ (**C**) and CD8+ (**D**) T cells from RA patients (n = 17) and healthy donors (n = 11) in response to pp65 peptide mixture or control peptide. **E,** Percentage of proliferated CD4+ and CD8+ T cells from RA patients (n = 7) and healthy donors (n = 6) incubated with total CMV lysate. In control cultures, a CMV-free control lysate did not induce significant proliferation (data not shown). **F,** Percentage of CD71+CD4+ T cells in cultures of PBMCs from RA patients (n = 13) and healthy donors (n = 7) incubated with either a pp65 peptide mixture or a control peptide. In **C–F,** data are shown as box plots. Each box represents the interquartile range. Lines inside the boxes represent the median. Lines outside the boxes represent the 10th and the 90th percentiles. $* = P < 0.05$ versus control peptide.

RA patients and healthy controls was detectable (Figure 1F). As expected, no significant T cell proliferation in response to pp65 was detectable in anti-CMV–seronegative RA patients or healthy controls (data not shown).

It has been shown that murine CMV infection leads to increased interleukin-6 production in mice, which in turn stimulates MCP-1 expression in and release from endothelial cells (18,19). Latent CMV infection in humans has not been reproducibly associated with a specific cytokine signature or an increased acute-phase response. In anti-CMV–seropositive patients with RA, however, cytokine production could be exaggerated by the superimposed chronic autoimmune response, with a consequent increase in the concentrations of proinflammatory mediators in anti-CMV–positive patients.

Analysis of the concentrations of IFNα and IP-10, 2 CMV-induced cytokines, revealed no difference in serum concentrations between anti-CMV–negative and anti-CMV–positive patients (data not shown). However, concentrations of MCP-1 were significantly elevated in anti-CMV–positive RA patients (median 89.4 pg/ml [IQR 53.6–150.5 pg/ml]) versus anti-CMV–negative RA patients (median 72.5 pg/ml [IQR 32.6–141.3 pg/ml]) ($P = 0.017$), suggesting that a prolonged state of endothelial cell activation might be associated with an aberrant immune response in anti-CMV–seropositive RA patients, or, alternatively, might be induced by the viral infection.

As a functional test of CMV-specific CD4+ T cells, their secretion of IFNγ in response to antigen-specific challenge with either pp65 peptide mixture or an irrelevant control peptide was determined in short-term cultures using an IFNγ secretion assay (Figure 2A). Only anti-CCP–positive patients were included. After in vitro stimulation with pp65 antigen, the frequencies of IFNγ-secreting CD4+ T cells in PBMCs from RA patients were higher than those in healthy controls (Figure 2B). Similar results were obtained when total CMV lysate was added to the cultures.

Association of anti-CMV IgG antibodies with more severe joint disease. Several previous studies have shown an association of an expanded CD4+CD28^{null} T cell compartment with a more severe clinical course of RA, in particular with extraarticular disease (3,4), accelerated atherosclerosis (20), and increased joint damage in early RA (21). The association of the frequency of CD4+CD28^{null} T cells with anti-CMV seropositivity observed in this study prompted us, therefore, to investigate the possible influence of anti-CMV seropositivity on the clinical severity of disease. Indeed, despite the almost identical frequency of anti-CMV IgG antibodies in patients and controls, anti-CMV seropositivity was found to have a profound impact on the clinical course of RA.

Analysis of the Steinbrocker stages assigned to the last available radiographs showed that joint destruction due to bone erosion was more severe in anti-CMV–positive patients, since the range was significantly higher

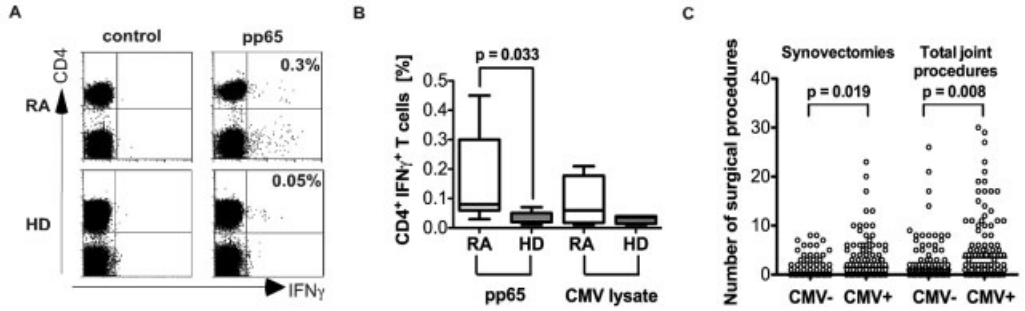

Figure 2. Anti-CMV–seropositive RA patients have increased frequencies of CMV-specific interferon-γ (IFNγ)–producing CD4+ T cells, and undergo joint surgery more frequently. PBMCs were incubated with either pp65 peptide mixture, CMV lysate, control peptide (control), or control lysate for 4 hours, and cytokine production was determined by IFNγ secretion assay followed by flow cytometric detection. **A,** Representative dot plots of CD4+IFNγ+ T cells in PBMCs from anti–cyclic citrullinated peptide (anti-CCP)–positive RA patients and healthy donors, incubated with pp65 or control peptide. Plots are gated on CD3+ live cells (by propidium iodide exclusion). **B,** Frequency of CD4+IFNγ production in response to pp65 peptide in cultured PBMCs from anti-CCP–positive RA patients (n = 7) and healthy donors (n = 7) and in response to CMV lysate in cultured PBMCs from anti-CCP–positive RA patients (n = 4) and healthy donors (n = 4). Data are shown as box plots. Each box represents the interquartile range. Lines inside the boxes represent the median. Lines outside the boxes represent the 10th and the 90th percentiles. **C,** Numbers of joint-related surgical procedures performed in anti-CMV–negative (n = 78) and anti-CMV–positive (n = 102) RA patients. Circles represent individual patients; horizontal lines indicate the median. See Figure 1 for other definitions.

Table 2. Comparison of patients with mild joint destruction and patients with severe joint destruction*

	Steinbrocker stage 0–II (n = 79)	Steinbrocker stage III or IV (n = 123)
Anti-CMV positive, no. (%)	37 (46.8)	78 (63.4)†
IgM-RF positive, no. (%)	68 (86.1)	112 (91.1)
IgM-RF titer	142 (46–520)	230 (84–623)
Anti-CCP positive, no. (%)‡	58 (77.3)	98 (84.5)
Anti-CCP titer	413 (51–1,367)	442 (92–1,600)
Shared epitope positive, no. (%)‡	29 (78.4)	61 (70.1)
DR4 shared epitope positive, no. (%)‡	20 (54.0)	43 (48.3)
C-reactive protein, mg/liter	11 (5–36)	19 (11–40)
No. of swollen joints	6 (2–14)	6 (2–10)

* Except where indicated otherwise, values are the median (interquartile range). Patients with stage 0–II disease according to the Steinbrocker criteria had mild joint destruction, and patients with stage III or IV disease according to the Steinbrocker criteria had severe joint destruction. See Table 1 for definitions.
† $P = 0.028$ versus patients with Steinbrocker stage 0–II disease.
‡ Data were not available for all patients.

in the anti-CMV–positive group than in the anti-CMV–negative group, as determined by nonparametric Mann-Whitney U test, although the overlap was extensive. (For both groups, the median stage was stage III, with an IQR of stage II to stage IV [$P = 0.044$]). In addition, the more severe joint destruction in anti-CMV–positive patients was illustrated by the more frequent occurrence of Steinbrocker stages III and IV in the anti-CMV–positive group compared to the anti-CMV–negative group (67.8% and 51.7%, respectively) ($\chi^2 = 4.738$; $P = 0.03$). The characteristics of the patients with mild joint destruction (radiographic stage 0–II disease according to the Steinbrocker criteria) versus those with severe joint destruction (stage III or IV disease according to the Steinbrocker criteria) are shown in Table 2.

Complete documentation of the history of joint-related surgical procedures was available for 78 anti-CMV–seronegative patients and 102 anti-CMV–seropositive patients. The aggravated joint disease in anti-CMV–positive patients was illustrated by the increased number of synovectomies required in this group, which was almost double that in the anti-CMV–negative group (median 1.5 [IQR 0–5] and 0 [IQR 0–2], respectively; $P = 0.019$) (Figure 2C). When joint replacement procedures and osteotomies were included in the retrospective analysis, the total number of joint-related surgical procedures was also higher in the anti-CMV–positive group than in the anti-CMV–negative group (median 3.5 [IQR 1–8] and 1 [IQR 0–5], respectively; $P = 0.008$) (Figure 2C).

In view of the older age of the anti-CMV–positive patients, the data were analyzed for a possible link between age, disease duration, and the degree of joint destruction, since increased cumulative damage to bone and joint structures could be the underlying cause of the observed differences. As expected, patient age was found to correlate with the disease duration prior to study inclusion ($R = 0.211$, $P = 0.00313$). Disease duration, in turn, was found to correlate closely with the observed radiologic damage and the total number of

Table 3. Multiple logistic regression model for the prediction of severe radiologic joint destruction (Steinbrocker stage III or IV)*

Independent variable	Coefficient	SE	*P*	OR (95% CI)†
Constant	1.362	0.698	0.051	3.902 (0.993–15.334)
Anti-CMV positivity	0.882	0.330	0.007	2.416 (1.266–4.610)
Age at disease onset	−0.0493	0.0118	<0.001	0.952 (0.930–0.974)
Anti-CCP positivity	0.567	0.716	0.429	1.763 (0.433–7.176)
RF positivity	0.452	0.579	0.435	1.572 (0.506–4.887)

* The presence or absence of severe radiologic joint destruction (either Steinbrocker stage III or IV or Steinbrocker stage 0–II, respectively) was used as the dichotomized, dependent variable in the logistic regression analysis (Hosmer-Lemeshow *P* value for goodness of fit 0.724, likelihood ratio 27.696 [$P < 0.001$]). 95% CI = 95% confidence interval (see Table 1 for other definitions).
† Odds ratio (OR) that a patient positive for the variable has disease more severe than Steinbrocker stage II.

Table 4. Multiple logistic regression model for the prediction of a history of >2 RA-related surgical procedures on bone and/or joint structures*

Independent variable	Coefficient	SE	*P*	OR (95% CI)†
Constant	0.845	0.760	0.266	2.328 (0.525–10.326)
Anti-CMV positivity	0.955	0.356	0.007	2.599 (1.293–5.228)
Age at disease onset	−0.0591	0.0133	<0.001	0.943 (0.918–0.968)
Anti-CCP positivity	0.504	0.626	0.421	1.655 (0.486–5.639)
RF positivity	0.532	0.802	0.507	1.702 (0.354–8.198)

* A history of either >2 or ≤2 RA-related surgical procedures on bone and/or joint structures was used as the dichotomized, dependent variable in the logistic regression analysis (Hosmer-Lemeshow *P* value for goodness of fit 0.834, likelihood ratio 30.344 [$P < 0.001$]). 95% CI = 95% confidence interval (see Table 1 for other definitions).
† Odds ratio (OR) that a patient positive for the variable has a history of >2 RA-related surgical procedures.

joint-related surgical procedures (R = 0.544, $P < 0.001$ and R = 0.637, $P < 0.001$, respectively).

In order to exclude the older age of the CMV-positive patients as a dominant confounding factor underlying the accelerated progression of joint destruction and the increased numbers of joint-related surgical procedures in these patients, the analysis was repeated after careful age-matching of anti-CMV–positive patients to anti-CMV–negative patients. To ensure stringent matching of anti-CMV–negative patients to anti-CMV–positive patients, age matching was performed by selecting patients from the total pool of analyzed anti-CMV–negative patients with an age difference of ≤6 months compared to the anti-CMV–positive patients. The difference in the number of RA-related surgical procedures on bone and/or joint structures between anti-CMV–positive and anti-CMV–negative patients remained significant when 62 anti-CMV–positive patients were compared to 62 age-matched anti-CMV–negative patients (median 4 [IQR 1–6] and 2 [IQR 0–5.75], respectively; $P = 0.024$). When synovectomies and tenosynovectomies alone were analyzed, their number also remained significantly higher in anti-CMV–positive patients than in anti-CMV–negative patients (median 2 [IQR 0–5] and median 0 [IQR 0–2], respectively; $P = 0.025$). In fact, 36.6% of the anti-CMV–negative patients had not had any joint-related surgical procedure, compared to only 16.4% of the anti-CMV–positive patients ($P = 0.03$).

Multiple logistic regression analysis reveals a significant influence of anti-CMV seropositivity and age at disease onset on joint destruction in RA. In order to analyze the contribution of the various clinical and serologic parameters examined to the severity of joint disease, multiple logistic regression analysis was performed. For this analysis, a dichotomized outcome parameter has to be entered as a dependent variable, which was achieved by introducing cutoffs for radiologic joint destruction (Steinbrocker stage >II) and for frequent joint surgery (>2 RA-related surgical procedures on bone and/or joint structures). When all relevant and available clinical parameters were entered into the regression analysis, the strongest predictive factor for severe joint destruction was disease duration, mirroring the progressive course of joint destruction in RA (data not shown).

If disease duration was not entered into the analysis, however, anti-CMV positivity and age at onset of disease exerted significant effects. When the variables anti-CCP positivity, IgM-RF positivity, age at disease onset, and anti-CMV seropositivity were analyzed as independent variables, anti-CMV seropositivity was the only parameter with an aggravating effect on both the presence of severe radiologic joint destruction (Table 3) and on the frequency of joint surgery (Table 4). The patient's age at the onset of disease also exerted a significant influence, but the effect size was minute and was associated with a negative correlation coefficient.

Further multiple logistic regression analysis of clinical parameters revealed no influence of anti-CMV positivity on the number of DMARD treatments initiated or treatment failures, steroid treatment, or disease activity at the time of analysis. RF and anti-CCP antibody levels were not increased in frequency or concentration in anti-CMV–positive patients.

DISCUSSION

This study was performed to investigate a possible link between latent CMV infection and the autoimmune disease RA, based on the hypothesis that the emergence of clonally expanded, $CD4+CD28^{null}$ T cells in both conditions indicates a possible pathogenetic link between them. No systematic analysis of the frequency

of anti-CMV seropositivity in RA has been published to date.

We showed that anti-CMV seropositivity by itself is not disproportionally common in RA. However, the observed higher frequency of anti-CMV seropositivity in older RA patients compared to healthy controls of the same age indicates that latent CMV infection might increase susceptibility to RA, at least in individuals older than 55 years of age. Anti-CMV seropositivity was previously shown to be associated with an expanded CD4+CD28^{null} T cell compartment in patients with RA (11,16), and this association was confirmed in the present study in a much larger patient cohort.

A direct comparison of the frequencies of CD4+ CD28^{null} T cells in RA patients to those in anti-CMV–seropositive or anti-CMV–seronegative healthy individuals was not possible in the present study, since only serum samples were available from the healthy blood donors. Of note, however, we and others have shown that even in anti-CMV–positive individuals, the median frequency of CD4+CD28^{null} T cells in healthy individuals younger than ~80 years of age does not exceed a fraction of 2.5% of circulating T cells (8,20,22,23), while those frequencies reach 10% and higher in RA patients (3,20,22–24). Accordingly, the median percentage of CD4+CD28^{null} T cells observed in the anti-CMV–positive RA patients in this study appears to exceed their frequency in anti-CMV–positive healthy controls by a factor of ~3. Therefore, although CD4+CD28^{null} T cells are expanded primarily in anti-CMV–positive individuals, they are further increased in RA patients, in particular in the presence of an extraarticular autoimmune phenomenon.

Loss of CD28 expression on CD4+ T cells has been shown to occur in 3 different scenarios. First, CMV infection of otherwise healthy individuals induces an increase in CD4+CD28^{null} T cells, for which no conclusive explanation has been put forward to date. Second, the increased CD4+CD28^{null} cell frequency in various autoimmune conditions (25–27) might at least partially be the result of chronic tumor necrosis factor exposure, which can contribute to the loss of CD28 by downregulating CD28 expression via inhibition of the activity of the CD28 minimal promoter (28). Finally, loss of CD28 expression seems to occur in CD4 lymphopenia, when extensive homeostatic proliferation is required to replenish the peripheral CD4+ T cell pool. This has been reported for recipients of allogeneic hematopoietic cell grafts (29) and in untreated patients with B cell chronic lymphocytic leukemia (CLL) (30), for which an expanded population of CD4+CD28^{null}, perforin-expressing T cells enriched for human CMV specificity (31) has been described.

Similar to those lymphopenic hematologic conditions, significant restrictions in the repertoire diversity and proliferative capacity of CD4+ T cells are also present in RA (2,14,32), with consequences such as repertoire disturbances, clonal expansions, and replicative exhaustion of the CD4+ T cell compartment. In view of the observed profound influence of anti-CMV seropositivity on the CD4+ T cell pool in RA patients, we propose that latent CMV infection adds severe additional strain on the already compromised T cell homeostasis in RA patients, thereby exaggerating the pathologic findings.

A proliferative response of CD4+ T cells toward CMV antigens as well as CMV-specific IFNγ production has previously been shown to be present in RA (33), but, unlike in the present study, it was not compared to that in healthy controls. In healthy individuals, some studies have described higher frequencies of IFNγ-secreting CD4+ T cells (24,34), but the findings of other studies were more compatible with our results (35,36). The use of a 4-hour short-term antigen-specific restimulation without additional costimulation and, in particular, the use of a highly specific cytokine secretion assay, along with the analysis of live cells, might have contributed to the low frequencies observed in both healthy controls and RA patients in our study. Of note, however, an RA-associated increase was detectable only in the frequency of IFNγ-secreting but not proliferating CD4+ T cells, which might reflect the restricted proliferative capacity of IFNγ-secreting, pathogenetically relevant CD4+ T cells described in RA (37).

It can be assumed that CMV-specific CD4+ T cells are preferentially CD4+CD28^{null}, as previously shown (24), and that they are likely to express inhibitory or activating KIR (9). In addition, CD4+CD28^{null} cells are known to produce excessive amounts of IFNγ, and our finding of an increased CMV-specific IFNγ response indicates that part of this CD4+CD28^{null} response might be driven by latent CMV infection.

The most important clinical finding of the present study is the influence of anti-CMV seropositivity on the radiographic progression of joint destruction, and in particular its highly significant association with the number of surgical procedures that were required. The aggravated joint disease in anti-CMV–positive patients is an indirect confirmation of the relevance of pathologic T cells to joint destruction in RA. Consistent with these findings, a prospective analysis has indeed shown that early in the course of RA, joint destruction already

progresses significantly faster in patients with expanded CD4+CD28^{null} T cells (21).

Very recently, clinical observations corresponding to our results have been reported for patients with B cell CLL (38). In that study and in the present study, the overall frequency of anti-CMV–seropositive patients did not differ from the frequency of anti-CMV–seropositive subjects in a healthy control group. However, consistent with our observations, anti-CMV–positive patients with B cell CLL not only had a markedly increased CMV-specific CD4+ T cell response comprising many CD4+CD28^{null} T cells but, more importantly, also exhibited an unfavorable clinical course with more infectious episodes and a reduction in overall survival by nearly 4 years. The authors speculate that in CLL patients, immunosuppression could trigger subclinical viremia that in turn activates and expands CMV-specific T cell populations (38). Similarly, DMARD therapy in RA could lead to an expansion of CMV-specific T cell clones, although CMV reactivation, i.e., viral DNA, was not detectable in the peripheral blood of the patients in this study (data not shown) or in the CLL patients, and no association between the number of consecutively prescribed DMARDs and anti-CMV seropositivity was detected in the present study.

An alternative explanation for the data on patients with B cell CLL was put forward in an accompanying editorial (39). It was suggested that the shortened life expectancy might result from indirect effects, either due to the dramatically expanded CMV-specific T cells themselves or due to the resulting constriction of the remaining T cell repertoire by those cells that compete for essential growth factors and for tissue niches required for T cell homeostasis (39). In addition, several potentially detrimental effects, including aberrant cytotoxicity and cytokine production, have been shown to occur in the RA T cell repertoire, which is characterized by restricted diversity (2,40).

We propose, therefore, that the exhaustive immune response due to latent CMV infection is more pronounced in RA patients than in healthy controls, leading to exaggerated immunosenescence, as indicated by the observed frequencies of clonal expansion and CD4+CD28^{null} cells. Premature immunosenescence, in turn, is associated with more severe joint destruction and extraarticular disease manifestations. Finally, the observed expansion of CD4+CD28^{null} T cells in patients with rheumatoid vasculitis suggests that latent CMV infection might even contribute to this serious condition (9). Further investigations in sufficiently powered, preferably prospective studies will be required for conclusive clarification of a potential link between latent CMV infection and susceptibility to RA.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Wagner had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Pierer, Baerwald.

Acquisition of data. Rothe.

Analysis and interpretation of data. Quandt, Schulz, Rossol, Scholz, Wagner.

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Latent Cytomegalovirus Infection in Rheumatoid Arthritis and Increased Frequencies of Cytolytic LIR-1+CD8+ T Cells

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Objective. **Leukocyte immunoglobulin-like receptor 1 (LIR-1) is up-regulated by cytomegalovirus (CMV), which in turn, has been associated with premature aging and more severe joint disease in patients with rheumatoid arthritis (RA). The aim of this study was to investigate the expression and functional significance of LIR-1 in CMV-positive RA patients.**

Methods. **We determined the phenotype, cytolytic potential, CMV-specific proliferation, and HLA–G–triggered, LIR-1–mediated inhibition of interferon-γ secretion of LIR-1+ T cells in RA patients and healthy controls.**

Results. **We found increased frequencies of CD8+ T cells with CMV pp65–specific T cell receptors in CMV-positive RA patients as compared to CMV-positive healthy controls. CMV-specific CD8+ T cells in these patients were preferentially LIR-1+ and exhibited a terminally differentiated polyfunctional phenotype. The numbers of LIR-1+CD8+ T cells increased with age and disease activity, and showed high levels of reactivity to CMV antigens. Ligation of LIR-1 with soluble HLA–G molecules in vitro confirmed an inhibitory role of the molecule when expressed on CD8+ T cells in RA patients.**

Conclusion. **We propose that latent CMV infection in the context of a chronic autoimmune response induces the recently described "chronic infection phenotype" in CD8+ T cells, which retains anti-infectious effector features while exhibiting autoreactive cytolytic potential. This response is likely dampened by LIR-1 to avoid overwhelming immunopathologic changes in the setting of the autoimmune disease RA. The known deficiency of soluble HLA–G in RA and the observed association of LIR-1 expression with disease activity suggest, however, that LIR-1+ T cells are insufficiently controlled in RA and are still likely to be involved in the pathogenesis of the disease.**

The human memory T cell compartment is shaped not only by antimicrobial immune responses, but also by autoimmunity and by latent infections with viruses such as cytomegalovirus (CMV) (1). The latter drive the generation of terminally differentiated T cells, which are characterized by the loss of costimulatory molecules such as CD27 and CD28, shortened telomeres, and by the expression of inhibitory natural killer (NK) cell receptors (2). CMV infection in immunocompetent hosts usually runs an asymptomatic course but has been reported to cause massive clonal expansions involving up to 40% of the global T cell pool (3). This increase over time in CMV-reactive T cells specific for antigens derived from latent CMV has been called memory inflation and involves both the CD4+ and the CD8+ T cell compartment (4,5). As a consequence, a stable CMV-reactive T cell compartment with an extremely dynamic cell turnover is established.

Clinically, CMV infection can cause organ-specific or systemic infections in immunocompromised patients. We and other investigators (6–8) have shown that the presence of a latent CMV infection influences the clinical course and outcome of rheumatoid arthritis (RA), the prototypical T cell–mediated autoimmune disease with severe perturbations of immune homeostasis, particularly in various T lymphocyte compartments. Similar observations have been reported in other autoimmune diseases, such as psoriasis (9), granulomatosis with polyangiitis (10,11), Alzheimer's disease (12), and systemic lupus erythematosus (13).

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Latent CMV infection has been associated with increased expression of the inhibitory NK cell receptor leukocyte immunoglobulin-like receptor 1 (LIR-1; also known as immunoglobulin-like transcript 2 and CD85j, with the gene name LILRB1) on CMV-reactive CD8+ T cells (14). LIR-1 belongs to a group of immunoregulatory receptors containing 2–4 immunoreceptor tyrosine-based inhibitory motifs within the cytoplasmic region. Upon tyrosine phosphorylation, LIR-1 recruits the SH2 domain–containing phosphatase 1 (SHP-1) tyrosine phosphatase or SH2 domain–containing inositol-5′-phosphatase (SHIP), both of which are involved in negative signaling and inhibition of cell activation (15). Furthermore, LIR-1 is expressed on almost all immune cells, including antigen-presenting cells and subsets of CD4+ and CD8+ T cells (16).

During the process of establishing latency following an acute CMV infection, the expression of LIR-1 on T cells is up-regulated (17,18), which results in reduced T cell proliferation in the autologous mixed lymphocyte reaction (19). The increase in LIR-1 expression after CMV infection is sustained throughout life and is regarded as a marker of premature immune senescence. It has been proposed that in otherwise healthy individuals, up-regulation of LIR-1 limits collateral tissue damage due to the sustained, long-term anti-CMV immune response (20), or it regulates T cell homeostasis (21). In conjunction with autoimmune conditions, however, LIR-1 expression appears to have additional and varying implications.

Diminished LIR-1 expression on B cells and altered functionality on T cells has been reported in systemic lupus erythematosus patients (22). Increased LIR-1 expression was found on the lymphocytes of patients with autoimmune thyroid disease (23) and multiple sclerosis (24). Genetic polymorphisms of LIR-1 were found to be associated with RA in patients not expressing RA-associated HLA–DRB1 alleles (25).

Since the effects of latent CMV infection and chronic immune response converge in patients with RA, we hypothesized that LIR-1 might be involved in the pathogenesis of the disease. The aim of this study, therefore, was to investigate the expression and functional significance of LIR-1 in CMV-positive RA patients. Based on the reported relevance of latent CMV infection to RA disease severity, we focused our study on the phenotype and function of polyfunctional and terminally differentiated CMV-specific lymphocytes positive for LIR-1, which is expressed not only on CD4+ T cells, but also, and more prominently, on CD8+ T cells.

PATIENTS AND METHODS

Patients and tissue samples. Patients with RA (n = 63) according to the American College of Rheumatology/European League Against Rheumatism 2010 criteria (26) were recruited from the rheumatology unit of the University of Leipzig. The control group consisted of age-matched healthy subjects (n = 70). The CMV status of the RA patients and healthy controls was determined by serologic analysis using enzyme-linked immunosorbent assay (ELISA; Medac). This study was approved by the local ethnics committee. Samples of synovium were obtained from patients undergoing synovectomy at the Department of Orthopedics at the University of Leipzig. Synovial tissue T cells were isolated as described previously (27).

Immunofluorescence staining and flow cytometry. T cell phenotyping was performed on freshly isolated peripheral blood mononuclear cells (PBMCs). The following antibodies were used in different fluorescent conjugates: anti-CD3 (BW264/56), anti-CD8 (BW135/80), anti-CD4 (M-T466), anti-CD28 (15E8), anti-CD27 (M-T271), anti-CD31 (AC128), anti-CD45RA (T6D11), anti-CD45RO (UCHL-1), anti-CCR7 (FR11-11E8), anti-CD57 (TB03), anti–programmed death 1 (anti–PD-1; PD1.3.1.3) (all from Miltenyi Biotec), anti–LIR-1 (292305 [R&D Systems] and GHI/75 [Miltenyi Biotec]), anti-IgG1 (11711 [R&D Systems] and IS6-11E5.11 [Miltenyi Biotec]), anti-CD3 (SP34-2; BD Biosciences), and anti–HLA–A2 (BB7.2; AbD Serotec).

Analysis of cells for the expression of surface markers was performed using FACSCalibur and LSR II flow cytometers (BD Biosciences). Data were analyzed with FlowJo software (Tree Star) and CellQuest (BD Biosciences) software. Doublets and dead cells were removed by exclusion of propidium iodide–positive cells.

Dextramer staining. PBMCs from RA patients were isolated by Ficoll-Paque density-gradient centrifugation. For determination of CMV-specific CD8+ T cells, PBMCs from CMV-positive RA patients and healthy controls were stained for HLA–A2 molecules. PBMCs (5×10^5) from HLA–A2–positive, CMV-positive RA patients (n = 8) and healthy donors (n = 12) were incubated with fluorescence-labeled monoclonal antibodies and an appropriate concentration of dextramer complexes for CMV proteins pp65 (CMV pp65/HLA–A*0201$_{\mathrm{NLVPMVATV}}$) and IE-1 (CMV IE1/HLA–A*0201$_{\mathrm{VLEETSVML}}$) (both from Immudex) in a small volume for 30 minutes at 4°C with protection from the light.

Cytotoxicity assay. Cytolytic CD8+ T cells were analyzed by cell surface mobilization of CD107a (lysosome-associated membrane protein 1). CD8+ T cells were isolated by positive selection using magnetic-activated cell sorting (Miltenyi Biotec). CD8-depleted PBMCs (5×10^6) were loaded with 5 μg of CMV pp65 peptide mixture or control peptide (15-mers, 11–amino acid overlap; Jerini Peptide Technologies) for 2 hours at 37°C. After washing, target cells and effector CD8+ T cells were seeded at an effector cell–to–target cell ratio of 4:1. Cytotoxicity assays were performed at 37°C for 4 hours in the presence of 0.0125 μg of CD107a antibody (Alexa Fluor 488–conjugated; BioLegend). Staphylococcal enterotoxin B (SEB; 1 μg/ml) (Sigma) was used as a positive control. Medium alone was used as an unstimulated control. After 1 hour of coculture, monensin (2 μM; eBioscience) or GolgiStop (2 μM; BD Biosciences) was added for the last 3 hours of cell culture. Subsequently, cells were stained and measured by fluorescence-activated cell sorting (FACS). Dead cells were removed by exclusion of propidium iodide–positive cells. Only experiments with >0.2% CD107a+CD8+ T cells were included in the statistical analysis.

Proliferation assay. The fluorescence-based proliferation analysis was performed by labeling PBMCs with 3 μg/ml of

5,6-carboxyfluorescein succinimidyl ester (Molecular Probes) or 10 μM Cell Proliferation dye eFluor 670 (eBioscience). Cells (2×10^6) were cultured for 7 days in the presence of 1 μg/ml of CMV lysate or control lysate (Microbix Biosystems) or 1 μg/ml of SEB (Sigma) as a positive control, in X-Vivo 15 medium (Lonza) containing 2 mM L-glutamine, 100 units/ml of penicillin, and 100 μg/ml of streptomycin.

Soluble HLA–G inhibition assay. The HLA–G positive choriocarcinoma cell line JEG-3 (HTB-36; ATCC) was stably transfected with the expression vectors of the HLA–G targeting microRNA-152 and with the respective mock vector (as a control), as previously described (28). Soluble HLA–G–containing and HLA–G–free cell culture supernatants were collected and stored at −80°C until time for use. A protein concentration step was applied, and the soluble HLA–G content was determined by ELISA (Exbio). For inhibition assays, CD8+ T cells were stimulated with 2 μg/ml of anti-CD3 for 6 hours in the presence of 5 μl of supernatant containing soluble HLA–G. Supernatant without soluble HLA–G was used as a control. Cells were subsequently stained and measured by FACS analysis. Dead cells were removed by exclusion of propidium iodide–positive cells. Intracellular staining of interferon-γ (IFNγ) was performed using an Inside Staining kit (Miltenyi Biotec).

Statistical analysis. GraphPad Prism 5.0 software was used for statistical analysis. Prior to all comparisons, a normality test was performed. Between-group differences in medians or means were analyzed by Student's *t*-test or the Mann-Whitney rank sum test as appropriate. Correlations were evaluated using Pearson's product-moment correlation or Spearman's rank correlation coefficient.

RESULTS

Analysis of CMV-specific CD8+ T cells in RA patients and controls. We reported previously that CMV-reactive CD4+ T cells, which secrete IFNγ in response to CMV antigen, are more frequent in RA patients than in healthy controls (7). To investigate the

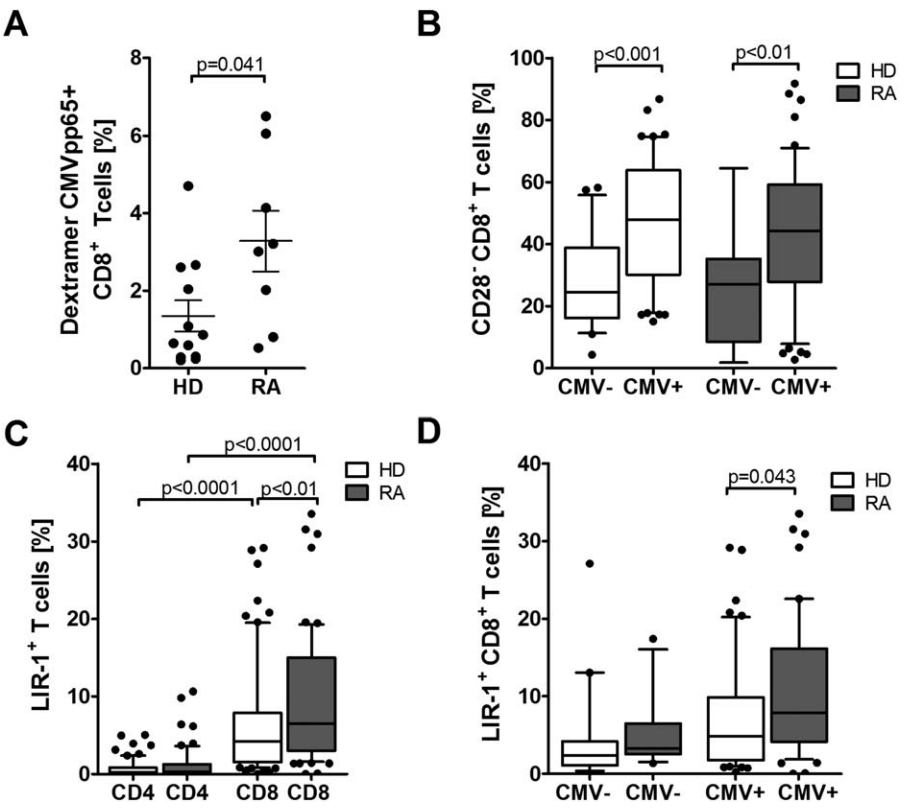

Figure 1. Increased frequencies of cytomegalovirus (CMV)–specific dextramer–positive CD8+ T cells and LIR-1+CD8+ T cells in rheumatoid arthritis (RA). **A,** Frequency of CMV pp65/HLA–A*0201$_{\text{NLVPMVATV}}$ dextramer–positive CD8+ T cells in healthy donors (HD; n = 12) and RA patients (n = 8). Each symbol represents an individual sample; horizontal lines and error bars show the mean ± SEM. **B,** Frequency of CD28– CD8+ T cells in CMV-negative and CMV-positive healthy donors (n = 8 and n = 57) and CMV-negative and CMV-positive RA patients (n = 20 and n = 50). **C** and **D,** Frequency of LIR-1+ T cells in CD4+ and CD8+ T cells from healthy donors (n = 63) and RA patients (n = 70) (**C**) and in CD8+ T cells from CMV-positive and CMV-negative healthy donors (n = 49 and n = 11) and RA patients (n = 51 and n = 19) (**D**). Data in **B–D** are shown as box plots. Each box represents the 25th to 75th percentiles. Lines inside the boxes represent the median. Whiskers represent the 10th and 90th percentiles. Solid circles indicate outliers. Except where indicated otherwise, frequencies are given as the percentage of total CD8+ T cells.

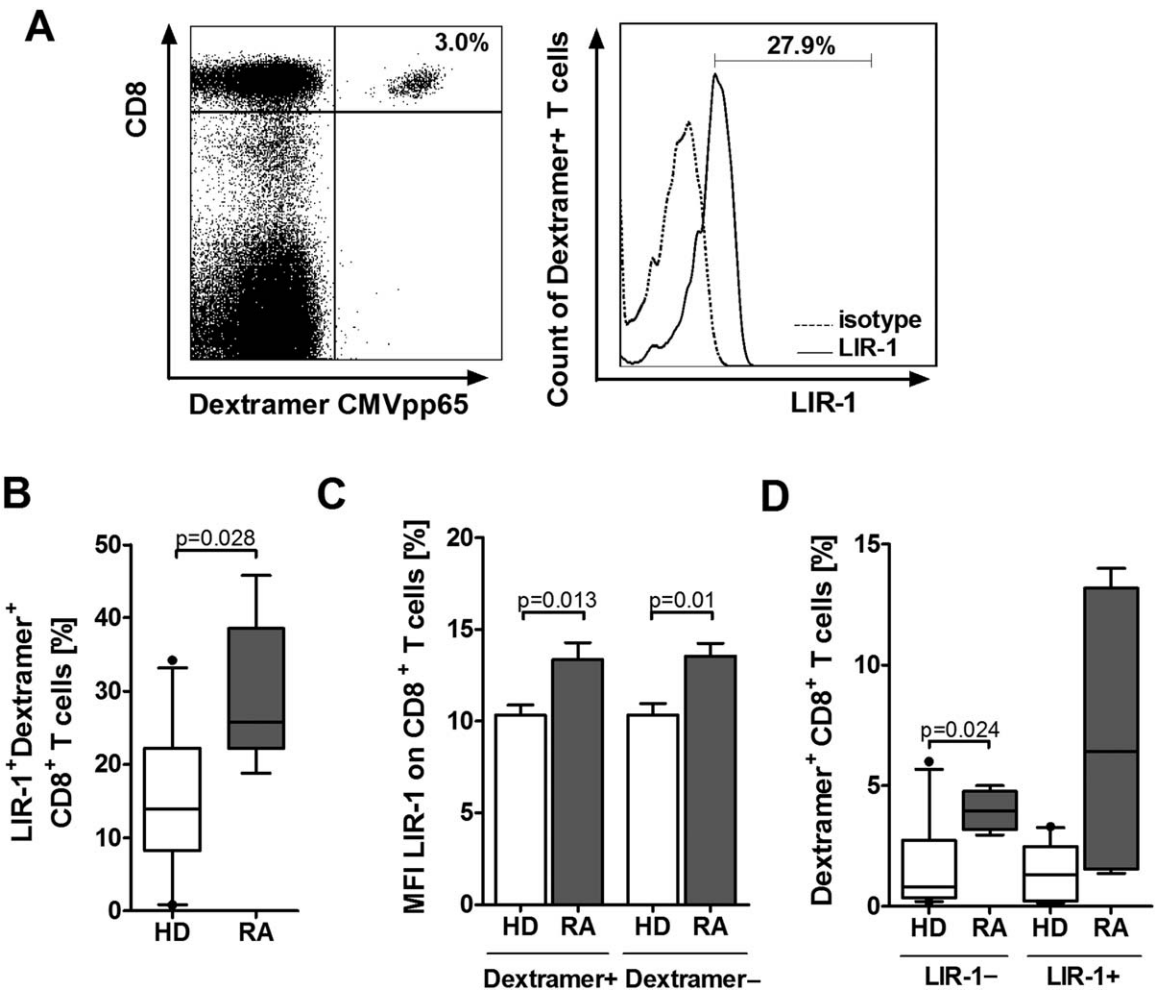

Figure 2. Increased frequencies of leukocyte immunoglobulin-like receptor 1 (LIR-1)–positive cells among cytomegalovirus (CMV)–specific dextramer–positive CD8+ T cells in rheumatoid arthritis (RA). **A,** Representative dot plot of dextramer CMV pp65 binding to CD8+ T cells (left) and representative histogram of LIR-1 expression on dextramer-positive CD8+ T cells (right). **B,** Frequencies of LIR-1+ cells among dextramer-positive CD8+ T cells from healthy donors (HD; n = 10) and from anti–cyclic citrullinated peptide–positive RA patients (n = 5). **C,** Fluorescence intensity of LIR-1 expression on dextramer-positive and dextramer-negative CD8+ T cells. **D,** Frequency of CMV pp65–specific dextramer–positive cells among LIR-1+ and LIR-1– CD8+ T cells from healthy donors (n = 10) and RA patients (n = 4). Values are the mean ± SEM. Data in **B** and **D** are shown as box plots. Each box represents the 25th to 75th percentiles. Lines inside the boxes represent the median. Whiskers represent the 10th and 90th percentiles. Solid circles indicate outliers.

consequences of latent CMV infection for the CD8+ T cell compartment in RA, the frequency of CMV-specific CD8+ T cells was determined using CMV pp65–specific dextramers and was found to be increased in RA patients positive for CMV and anti–cyclic citrullinated peptide as compared to CMV-positive healthy controls (Figure 1A). Latent CMV infection is known to cause memory inflation, with a concomitant increase in polyfunctional, terminally differentiated CD4+ and CD8+ T cells with pathologic phenotypes (5,29). As an example of such a phenotype, we analyzed the frequencies of CD28– T cells and found an increase in both the CD4 and the CD8 T cell compartments in CMV-positive as compared to CMV-negative RA patients (Figure 1B).

Analysis of LIR-1+CD8+ T cells in RA patients and controls. CMV infection is known to induce overexpression of LIR-1. In RA patients and healthy control subjects, CD8+ T cells express LIR-1 more frequently than do CD4+ T cells, as previously reported (20) and as confirmed by the findings of our present study (Figure 1C). Surprisingly, LIR-1 expression on CD8+ T cells in RA patients was even higher than that in healthy controls. This increase was most pronounced in CMV-positive RA patients as compared to CMV-positive controls (Figure 1D).

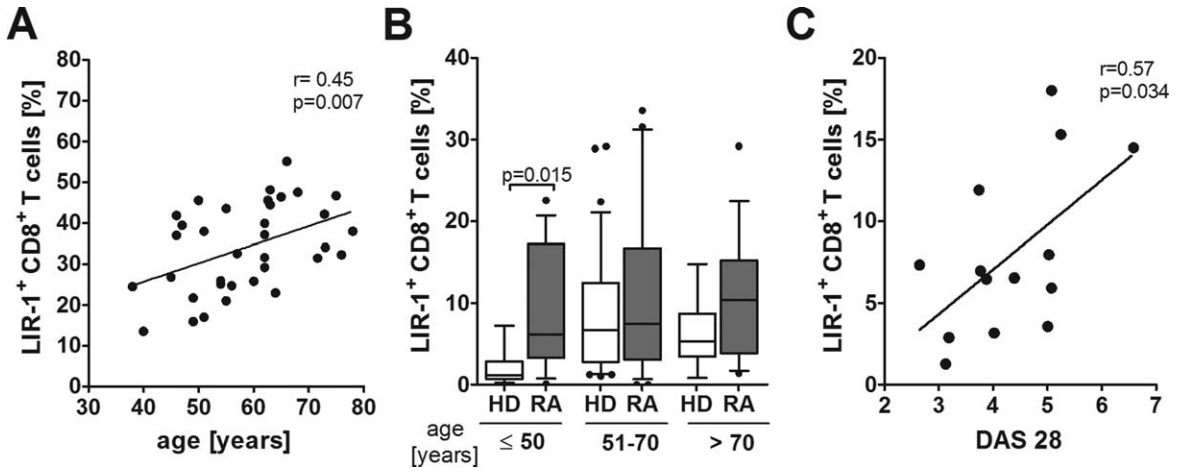

Figure 3. Influence of age and disease activity on frequencies of LIR-1+CD8+ T cells in rheumatoid arthritis (RA). **A,** Correlation of the frequencies of LIR-1+CD8+ T cells in the peripheral blood of 35 RA patients by age group. **B,** Frequency of LIR-1+CD8+ T cells in the peripheral blood of RA patients and healthy donors (HD) in 3 age groups: ≤50 years (n = 13 and n = 8, respectively), 51–70 years (n = 24 and n = 37, respectively), and >70 years (n = 16 and n = 7, respectively). Data are shown as box plots. Each box represents the 25th to 75th percentiles. Lines inside the boxes represent the median. Whiskers represent the 10th and 90th percentiles. Solid circles indicate outliers. **C,** Correlation of the frequencies of LIR-1+CD8+ T cells in the peripheral blood of 14 RA patients by Disease Activity Score in 28 joints (DAS28). All frequencies are given as the percentage of total CD8+ T cells.

In CMV-negative RA patients, a trend toward increased frequencies of LIR-1+CD8+ T cells was discernible, but the difference did not reach statistical significance ($P = 0.085$).

LIR-1+CD8+ T cells were also quantified in the affected joints of RA patients and were found in significant numbers, both in rheumatoid synovium and in synovial fluid, although the frequencies in the synovial membrane were lower than those in the peripheral blood (*P* not significant) (data not shown).

Analysis of LIR-1 expression on CMV pp65–specific CD8+ T cells revealed increased LIR-1+ cells among dextramer-positive CD8+ T cells from RA patients as compared to controls (Figures 2A and B). Importantly, LIR-1 expression levels were also significantly higher on dextramer-negative CD8+ T cells from RA patients as compared to controls (Figure 2C). In RA patients, more CD8+ T cells were CMV pp65 specific, both among LIR-1+ and LIR-1– cells (Figure 2D). Determination of CD8+ T cells specific for CMV IE-1 revealed very low frequencies, both in RA patients and in healthy individuals (data not shown).

The frequencies of LIR-1+CD8+ T cells were found to increase with age (Figure 3A), as reported previously (18). Of note, however, the frequencies of LIR-1+CD8+ T cells were also higher when only subjects younger than age 50 years were compared (Figure 3B). Clinically, increased frequencies of LIR-1+CD8+ T cells were found to be associated with higher levels of disease activity, as indicated by a significant positive correlation with the Disease Activity Score in 28 joints (Figure 3C).

Phenotyping of LIR-1+CD8+ T cells. Repeated chronic immune responses often result in polyfunctional, terminally differentiated, and possibly exhausted T cell phenotypes, such as the CD4+CD7–CD28– T cell subset originally described in RA (30) or the CD8+CD27−CD28− T cell subset (1). Phenotype characterization of LIR-1+CD8+ T cells from RA patients by flow cytometry revealed them to be preferentially CD27–CD28– and CD28–CD57+ and to contain increased frequencies of CD45RA+CCR7– effector T cells. Furthermore, LIR-1+CD8+ T cells more frequently express the NK cell marker CD56 than do LIR-1– T cells. Analysis of the "exhaustion" marker PD-1 revealed no difference in its expression on LIR-1+CD8+ T cells as compared to LIR-1– T cells (Figure 4A). Expression of the fractalkine receptor CX_3CR1, which enables T cells to migrate into the rheumatoid synovium (31), was also increased on LIR-1+CD8+ T cells from RA patients as compared to LIR-1–CD8+ T cells from RA patients and from healthy controls (Figure 4B). The chemokine receptor CCR5, which is also associated with latent virus infection and an effector phenotype of T cells (32), was not differentially expressed on LIR-1+ T cells from RA patients as compared to healthy controls (Figure 4C).

Functional characterization of LIR-1+CD8+ T cells in RA. Inhibitory immune receptors, such as LIR-1, are expressed on T cells, where they control the magnitude of the immune response after activation. We therefore analyzed the regulation of LIR-1 expression in vitro, the

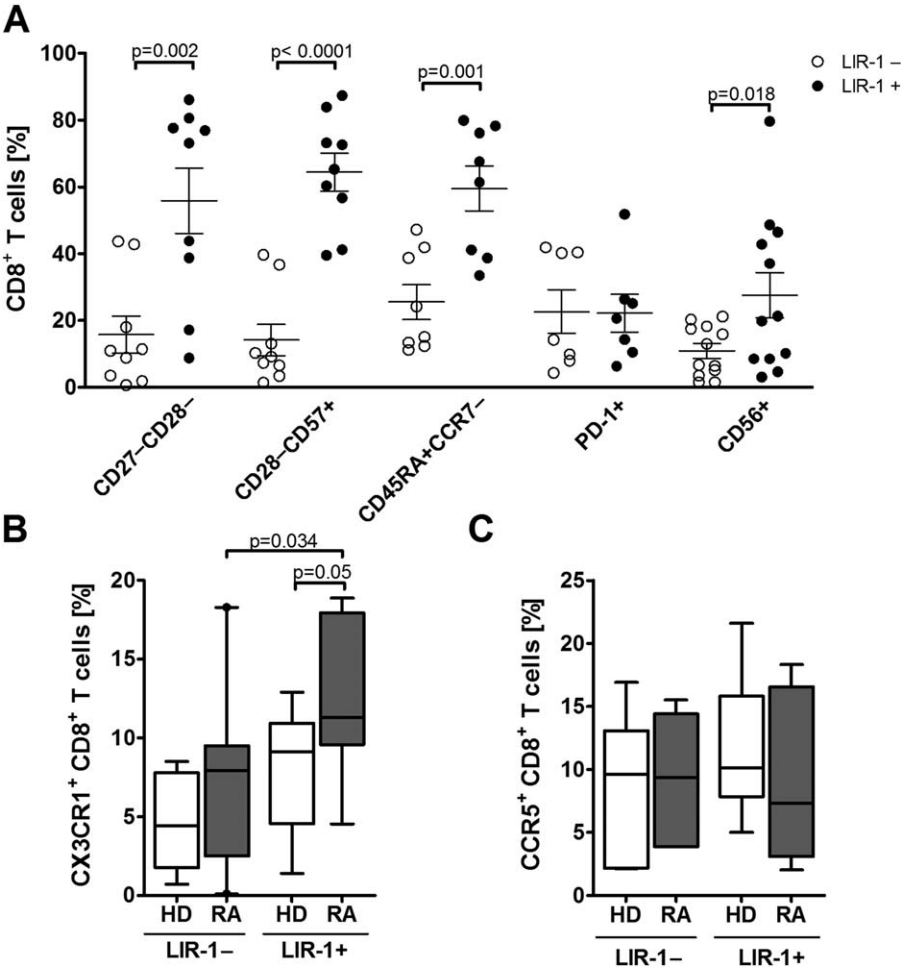

Figure 4. Phenotypic characterization of LIR-1+CD8+ T cells in rheumatoid arthritis (RA). **A,** Frequencies of CD27–CD28– T cells, CD28–CD57+ T cells, CD45RA+CCR7– T cells, programmed death 1 (PD-1)–positive T cells, and CD56+ T cells in LIR-1+CD8+ and LIR-1–CD8+ T cell subsets in RA patients. Each symbol represents an individual sample; horizontal lines and error bars show the mean ± SEM. **B** and **C,** Frequencies of chemokine receptors CX_3CR1+ (**B**) and CCR5+ (**C**) among LIR-1+CD8+ T cells and LIR-1–CD8+ T cells from RA patients (n = 9) and healthy donors (HD; n = 8). Data are shown as box plots. Each box represents the 25th to 75th percentiles. Lines inside the boxes represent the median. Whiskers represent the 10th and 90th percentiles. Solid circle indicates an outlier. LIR-1 = leukocyte immunoglobulin-like receptor 1.

effector status of LIR-1+CD8+ T cells, and the inhibitory effect of the LIR-1 molecule on CD8+ T cells from RA patients.

To gain insight into the regulation of LIR-1, PBMCs from RA patients were stimulated in vitro for 6 days using various stimuli. The expression of LIR-1 on CD8+ T cells was down-regulated after 2 days without exogenous stimuli. Expression could be maintained for 4 days with anti-CD3, and it remained detectable even after 6 days of culture in the presence of CMV lysate or phytohemagglutinin (data not shown).

Functionally, the cytolytic potential of LIR-1+CD8+ T cells was determined by CD107a mobilization assay using flow cytometry. Significantly higher expression of CD107a in response to CMV pp65–loaded PBMCs was detected on CD8+ T cells from RA patients as compared to healthy controls, indicating increased CMV-specific cytolytic potential (Figures 5A and B). Control peptide–loaded antigen-presenting cells could not induce CD107a expression in CD8+ T cells (data not shown). In healthy controls and RA patients, increased cytolytic potential was mainly found in LIR-1+CD8+ T cells.

The proliferative potential of LIR-1+CD8+ T cells after stimulation with CMV lysate was also quantified in vitro (Figures 5C and D). LIR-1+CD8+ T cells were not found to be proliferatively exhausted, since

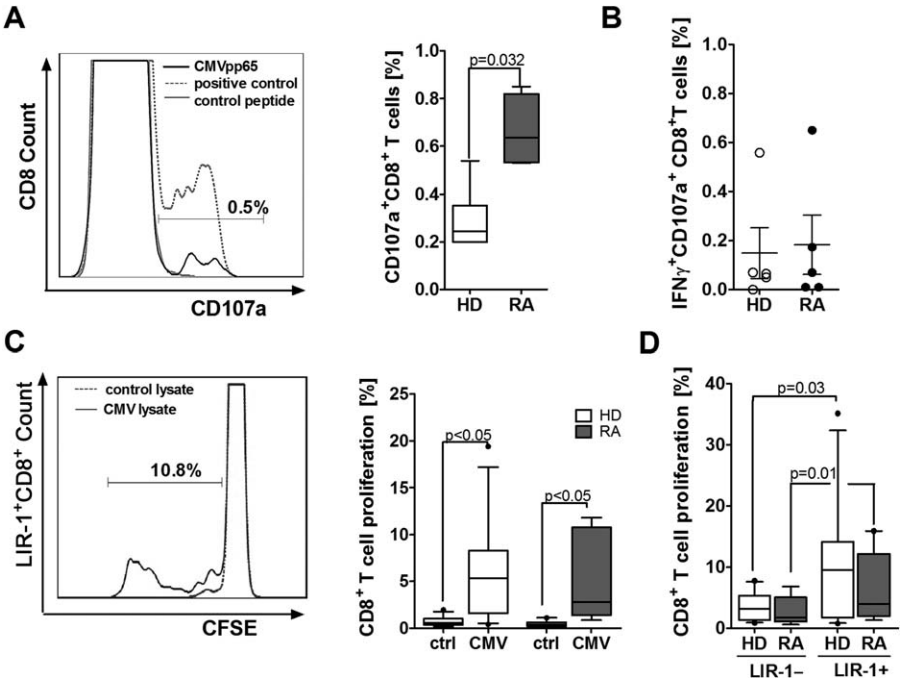

Figure 5. Functional analysis of LIR-1+CD8+ T cells in rheumatoid arthritis (RA). **A** and **B,** CD107a-degranulation assay. The representative histogram in **A** shows CD107a expression on CD8+ T cells after restimulation with cytomegalovirus (CMV) pp65 peptide–loaded, staphylococcal enterotoxin B (SEB; positive control)–loaded, or control peptide–loaded antigen-presenting cells (left). The percentage of CD107a+CD8+ T cells is also shown (right). In **B**, the percentage of IFNγ+CD107a+CD8+ T cells from RA patients (n = 5) and healthy donors (HD; n = 5) is shown. **C** and **D,** Proliferation of LIR-1+CD8+ T cells from CMV-positive healthy donors and RA patients. The representative histogram in **C** shows proliferation of LIR-1+CD8+ T cells after restimulation with CMV lysate (1 μg/ml) or control lysate (left). The proliferation rate of CD8+ T cells incubated with CMV lysate or control lysate from healthy donors (n = 12) and patients with RA (n = 8) is also shown (right). In **D**, the percentage of proliferated LIR-1–CD8+ and LIR-1+CD8+ cells from healthy donors (n = 12) and RA patients (n = 8) in response to CMV lysate is shown. Data in **A** (right), **C** (right), and **D** are shown as box plots. Each box represents the 25th to 75th percentiles. Lines inside the boxes represent the median. Whiskers represent the 10th and 90th percentiles. Solid circles indicate outliers. In **B**, each symbol represents an individual sample; horizontal lines and error bars show the mean ± SEM.

they mounted a robust proliferative response to CMV lysate, which exceeded the proliferation rates of LIR-1–CD8+ T cells both in RA patients and in healthy controls (Figure 5D). Despite their enhanced cytolytic potential, however, CD8+ T cells from RA patients did not show an increased proliferation rate as compared to healthy controls (Figure 5C).

Soluble HLA–G is a ligand of LIR-1 and is known to evoke an inhibitory signal in most cell types. In patients with RA, decreased serum levels of soluble HLA–G have been reported (33), which may contribute to autoimmunity. Hence, we investigated the responsiveness of RA CD8+ T cells by triggering LIR-1. Addition of exogenous soluble HLA–G in vitro led to decreased frequencies of CD107a+CD8+ T cells (Figures 6A and B), indicating that the inhibitory signal triggered by ligation of LIR-1 would be effective if sufficient soluble HLA–G molecules were available in RA. Furthermore, intracellular IFNγ expression in CD8+ T cells was also down-regulated in cultures with soluble HLA–G (Figure 6C).

DISCUSSION

Latent CMV infection and RA share several phenotypical features in the T cell compartment. In addition, the clinical impact of CMV infection on the RA disease course has previously been reported (6–8). We describe herein a population of CD8+ T cells in CMV-positive RA patients that exhibits proinflammatory, cytolytic, and antiviral features and is functionally inhibited due to up-regulated expression of LIR-1.

In healthy individuals, expression of LIR-1 on CD8+ T cells is up-regulated by CMV infection, possibly with the goal of limiting collateral tissue damage due to the longstanding immune response against the latent virus or, alternatively, as a homeostatic mechanism (6–8). We found that the frequency of LIR-1+CD8+ T cells was significantly higher in CMV-positive RA patients than in CMV-positive healthy controls and that it increased not only with age, but also with higher levels of disease activity. In addition, LIR-1 was up-regulated on CD8+ T cells that were not specific for CMV antigens.

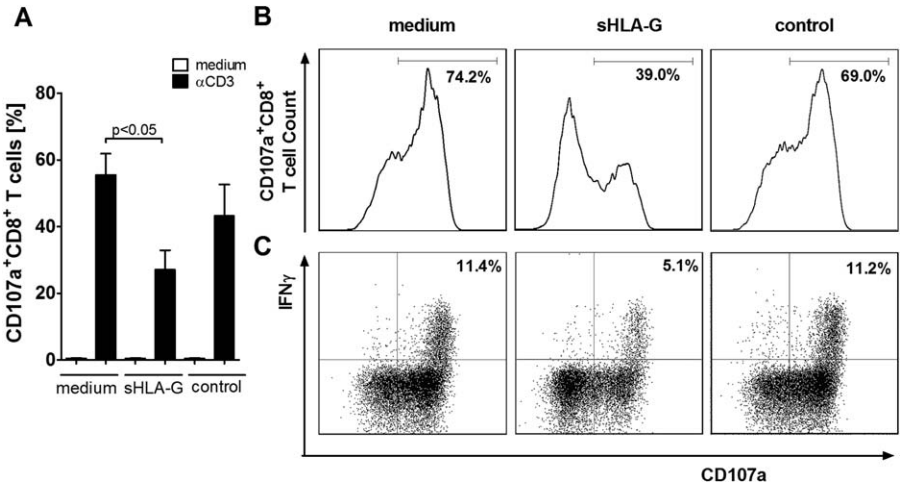

Figure 6. Inhibition of cytotoxic CD8+ T cells in patients with rheumatoid arthritis (RA) by soluble HLA–G (sHLA–G). **A,** Inhibition of CD107a expression after the addition of medium alone, soluble HLA–G (5 μl), or anti-CD3 control to in vitro cultures of CD8+ T cells. Values are the mean ± SEM of 3 samples per group. **B** and **C,** Representative histograms showing the frequencies of CD107a+CD8+ T cells (**B**) and the expression of intracellular interferon-γ (IFNγ) on CD8+ T cells (**C**) from RA patients after in vitro activation in medium or soluble HLA–G–containing or control supernatants.

In healthy individuals, lymphocytes involved in memory inflation tend to acquire a specific phenotype, which was previously described as "exhaustive." More recently, it has been suggested that this "chronic infection phenotype" represents a population of T cells that can still efficiently control latent infection, while certain levels of effector function are diminished to prevent overwhelming immunopathologic changes due to collateral autoreactivity (34). Increased LIR-1 expression on CD8+ T cells is likely to represent such a CMV-induced chronic infection phenotype, since it is linked to latent CMV infection in healthy individuals (18). Our results show that in comparison to healthy controls, LIR-1 was further up-regulated on CD8+ T cells in RA patients. In this autoimmune disease, increased LIR-1 expression could result from an insufficiently controlled latent CMV infection, leading to higher numbers of T cells, which are required and recruited, or it could represent a regulatory mechanism aimed at controlling autoimmunity in RA in the context of latent CMV infection.

The functional analysis confirmed that LIR-1+ T cells in RA are polyfunctional and have cytolytic potential. Their higher expression of CD56 and CD57, which is associated with increased cytotoxicity in CMV-seropositive healthy individuals (1,35), might further increase their cytolytic potential in RA. Phenotype analysis using the T cell differentiation markers CCR7 and CD45RA confirmed that LIR-1+CD8+ T cells are effector T cells. In addition, their expression of CX_3CR1, which is known to be up-regulated on CMV-specific CD4+ and CD8+ T cells from healthy individuals (36), might enable them to migrate toward fractalkine gradients, which has been reported to occur in the rheumatoid synovium (31). Differences in PD-1 expression between LIR-1+ and LIR-1– CD8+ T cells were not significant.

In phases of CMV reactivation or of relevant suppression of the controlling immune response, the CMV-specific LIR-1+CD8+ T cells appear to be able to mount a cytolytic immune response. Numerically, only 25% of the CMV pp65–specific CD8+ T cells are LIR-1+, and <10% of LIR-1+ cells are specific for the immunodominant CMV antigen pp65. Even assuming that another 10% of LIR-1+ cells recognize other CMV antigens, it still leaves the majority of LIR-1+CD8+ T cells unreactive to CMV. The observation that LIR-1 overexpression is also found on T cells that are not reactive to CMV antigens and possibly even on T cells from CMV-negative RA patients indicates that CMV-independent factors related to the chronic autoimmune disease are also involved.

Our finding of increased proliferative activity is evidence against the interpretation that expression of LIR-1 on CD8+ T cells is the equivalent of T cell exhaustion, since T cell exhaustion is generally regarded to be accompanied by low proliferative capacity (37,38). Our observation is consistent with a similar study showing increased proliferative capacity of LIR-1+CD8+ T cells from healthy controls (14). In RA, exaggerated proliferative replication of LIR-1+CD8+ T cells could even contribute to the observed increase in the frequency of CMV-specific dextramer–positive CD8+ T cells, possibly due to a failure to "switch off" ongoing immune responses. Alternatively, the increased frequency of CMV-specific T cells could be a

consequence of globally increased T cell proliferation leading to replicative senescence in this disease (39), but this latter explanation is partially contradicted by the increased proliferation among LIR-1+CD8+ T cells.

LIR-1 remains functional as an inhibitory receptor in RA, since we found LIR-1 ligation by soluble HLA–G to block cytotoxicity in our experiments. Antigen recognition by CD8+ T cells requires antigen-presenting major histocompatibility complex (MHC) class I molecules to interact with T cell receptors and CD8 coreceptors within the tightly organized and spatially focused immunologic synapse. Those MHC class I molecules are ligands for LIR-1, and when LIR-1 is recruited to the immunologic synapse, it exerts a strong inhibitory effect, even more so if it encounters the viral high-affinity ligand UL18, which is expressed on the juxtaposed cells in latent CMV infection (40). Accordingly, up-regulation of the inhibitory receptor LIR-1 in RA could be an attempt to limit autoreactivity in order to alleviate the autoimmune disease.

Soluble HLA–G has been reported to be reduced in RA (33) and has been implicated in the pathogenesis of RA by the associations of its genetic polymorphisms with disease susceptibility (41). Latent CMV infection down-regulates HLA–G expression (42), and decreased levels of soluble HLA–G, in turn, could cause LIR-1+CD8+ T cells in RA to become hyperactive.

Taken together, our data suggest that the intricate network and finely tuned crosstalk of UL18, HLA–G, and classic MHC class I molecules with LIR-1 is disturbed during the course of a CMV infection in RA. As a consequence, deficiency of soluble HLA–G might diminish the inhibitory effects of LIR-1 on CD8+ T cells in CMV-positive RA patients. LIR-1+CD8+ T cells, in turn, could also be involved in the pathogenesis of RA by contributing directly to chronic inflammation. The observed significant correlation of the frequency of LIR-1+CD8+ T cells with disease activity strongly supports this hypothesis. At the same time, those cells might still be involved in the immunologic control of the latent CMV infection, which illustrates possible unwanted side effects of immunosuppression in this disease.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Wagner had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Rothe, Quandt, Pierer, Wagner.

Acquisition of data. Rothe, Schubert, Rossol, Klingner, Jasinski-Bergner, Scholz, Seliger, Pierer, Baerwald, Wagner.

Analysis and interpretation of data. Rothe, Quandt, Schubert, Seliger, Wagner.

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Peripheral CD4CD8 Double Positive T Cells with a Distinct Helper Cytokine Profile Are Increased in Rheumatoid Arthritis

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Abstract

Peripheral CD4CD8 double positive (DP) T cells have been reported to play a role in several autoimmune diseases, virus infections and cancer. In rheumatoid arthritis (RA), both CD4 and CD8 single positive (SP) T cells are known to be involved in the pathogenesis, but the role of peripheral CD4CD8 DP T cells has not been investigated in detail. Anti cyclic citrullinated antibodies (ACPA) positive and ACPA negative RA patients, patients with systemic lupus erythematodes (SLE) and age matched healthy donors (HD) were enrolled in the analysis. The frequencies and phenotype of DP T cells in PBMC were investigated. In addition, DP T cells were quantified in biopsies from rheumatoid synovium. After in vitro restimulation, the cytokine production of DP T cells was investigated in cultures of PBMC. CMV specific cytokine secretion as well as proliferation was analyzed following antigen specific restimulation after an appropriate culture duration. DP T cells were found more frequently in RA patients than in healthy controls or patients with SLE. These DP T cells express αβ TCRs, are of a memory phenotype and share features of both CD4 as well as CD8 SP T cells. Importantly, DP T cells were found to also be present in the rheumatoid synovium. Further characterization of DP T cells from RA patients revealed increased production of IL-21 and IL-4, implying a possible role as T helper cells. In addition, DP T cells in RA seem to contribute to the inflammatory process, because they produce significantly more IFNγ than counterparts from HD and are increased in CMV+ RA patients. Given their capacity to produce a variety of cytokines (IL4, IL21 and IFNγ), their association with ACPA positive RA and their presence in the synovium, we suggest an important role of double positive T cells in the pathogenesis of rheumatoid arthritis.

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Competing Interests: The authors declare that they have no competing interest.

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Materials and Methods

Patients and Healthy Individuals

A total of 59 RA patients according to the 2010 EULAR/ACR criteria (female: 46, male: 13, mean age 59.4 years, range 34–79 years) were recruited, among them 39 $ACPA^+$ and 20 $ACPA^-$ patients. 39% of the RA patients were treated with biologicals in combination with conventional standard therapy. Sex and age distribution in $ACPA^+$ versus $ACPA^-$ patients was similar. In addition, 8 SLE patients (all female, mean age 44.3 years, range 21–54 years) were included. Blood of 36 HD (female: 21, male: 15, mean age of 57.1 years, range 25–71 years) who never had evidence of a chronic inflammatory disorder were recruited as controls. The 4 RA patients undergoing knee surgery (2 male, 2 female) were all $ACPA^+$.

Ethics Statement

Written consents were obtained from all patients and healthy donors. The local ethics committee of the University of Leipzig approved the study.

Antibodies and Reagents

RPMI 1640 was from Lifetechnologies. X-Vivo15 media was supplied by Lonza. aCD3, aCD4, aCD8 (recognizing the α chain), aCD28, aCD45RO, aCD56, aCCR7, a-IL17, aTCRα24-Jα18 (clone: 6B11), cytokine secretion assays for IFNγ and IL-4, a-fibroblast microbeads and Cytostim were purchased from Miltenyi. Collagenase, Hyaluronidase and DNAse were all from Sigma-Aldrich. aCD45 and aCD38 were from Immunotools. CFDA-SE was purchased from Molecular Probes/Invitrogen. Intra staining Kit, aCD16, aCD8β and aCD3 were from Beckton Dickinson. aCXCR5 was supplied by R&D Systems and aIL21 was from ebioscience. The Beta Mark TCRVβ Repertoire Kit was supplied by Beckman Coulter. The antibodies were used in different conjugates of FITC, PE, PerCp, APC, APC-Vio770 and PE-Cy7.

PBMC Generation and FACS Analysis *ex vivo*

PBMCs were isolated from EDTA whole blood or buffy coats. Plasma was always discarded from whole blood samples prior to Ficoll-gradient for PBMCs isolation. Subsequently a erythrocyte lysis step with lysis-buffer was applied. Cells were stained with

different antibodies and kept on ice throughout the assay. Live Cell analysis (use of PI) with doublet exclusion (LSR II) were performed on a FACS Calibur ™ or a LSR II (both Beckton Dickinson) using Cellquest, FACS DIVA and FlowJo software.

CMV Specific Cytokine Production and Proliferation

These assays were performed as described recently. [1] In brief, 1×10^6 PBMC were CFDA-SE labeled and cultured for 7 days (proliferation) or left unlabeled and cultured for 4 hrs (2×10^6, IFNγ secretion) in the presence of CMV lysate/control lysate (Microbrix Biosystems Inc) of 3 μg/ml in 24-well plates in X-VIVO 15 medium.

Short Term Culture and Staining for Cytokine Analysis

PBMCs were cultured in X-Vivo 15 supplemented with 1% of each glutamin and penicillin/streptomycin in a density of 5×10^6 for cytostim (1:50) or 3×10^6 for PMA (20 ng/ml and Ionomycin (0.5 μg/ml). Culture time was 4 hrs for both and Monensin (2 μM) was added to the last 3 hrs of PMA/Ionomycin cultures. Cytokines were either detected with cytokine secretion assays (IFN-γ and IL-4) following the manufactures protocol by Miltenyi or by intracellular staining (IL-21 and IL-17) using an intra staining Kit.

Tissue Digestions and Leucocyte Extraction

Synovial biopsies from RA patients undergoing surgery were obtained and leucocyte isolation was performed as follows. Tissue was cut into pieces and incubated with an enzyme solution (collagenase, hyaluronidase, DNAse in RPMI) for 90 min and 37° under constant rotation. Single cell suspension was obtained using gauze and smooth mechanical disruption of digested tissue. Subsequently cells were sorted for non-fibroblasts using anti-fibroblast microbeads from Miltenyi. Non-fibroblast were used for FACS analysis and CD45 staining was used additionally to other antibodies in order to discriminate non-leucocytes.

Statistics

Statistical evaluation was performed using Prism version 3.0cx software. Mann-Whitney test, unpaired student's t-test and correlation analysis with spearmen were applied.

Introduction

Peripheral CD4CD8 double positive (DP) T cells have first been identified more than 20 years ago. Like their progenitors in the thymus, they express the coreceptors CD4 and CD8 simultaneously, but in contrast to immature double positive thymocytes, they show varying degrees of coreceptor expression, and display a memory phenotype but no markers of recent thymic emigrants [2–4]. DP T cells can be found in the blood of healthy individuals where they account for about 1% of all T cells within PBMCs, but are also present in the skin of melanoma patients and in systemic sclerosis [5,6]. During the cause of severe virus infections such as HIV and Hepatitis, increased frequency of DP T cells have been described, which are Ag-specific and of high effector potential. [2,7]. DP T cells can provide B cell help both by production of appropriate cytokines and by cell contact due to their T helper like phenotype [5,6], but they can also acquire killer like capacity [7,8].

In rheumatoid arthritis, CD4+ Th cells play a pivotal role in the pathogenesis, as indicated by numerous genetic associations of the disease with polymorphisms in T cell related genes as well as by the clinical efficacy of CD28 co-stimulation [10] and CD4 co-receptor blockade [9] [11]. The peripheral CD4+ T cell pool in RA is characterized by several alterations including a paucity of naïve T cells and recent thymic emigrants, an increased memory pool and a global loss of T cell receptor diversity accompanied by large clonal expansions [12,13].

B cell autoreactivity is also essential in RA pathogenesis, as indicated by autoantibody production and by the clinical efficacy of B cell depletion [14]. The T cell help required for this has been suggested to involve CD40-CD40L interactions [15], but additionally other receptors such as BAFF-R [16] have been identified more recently. The result is the production of disease relevant autoantibodies like anti-citrullinated peptide antibodies (ACPA), anti-RA33, RF and others. ACPA positive RA in particular has been associated with more severe joint destruction [1], frequent extraarticular manifestations and enhanced subclinical artheriosclerosis [17].

A possible role of CD4CD8 DP T cells in the pathogenesis of RA has not been investigated.

Here we report, that CD4CD8 double positive T cells are expanded in the peripheral blood in $ACPA^+$ RA and can also be found in the rheumatoid synovium. DP T cells in RA show features of T helper cells by the production of IL-4 and IL-21. Interestingly, CMV^+ RA patients show increased frequencies of these cells and their number correlates positively with CMV specific IFNγ producers. In line with this, a high number of these cells have lost the CD28 costimulator and DP T cells of RA patients show higher amounts of IFNγ producers than HD counterparts. Our data are the first demonstrating a role for CD4CD8 double positive T cells in RA.

Results

$ACPA^+$ RA Patients have Increased Frequencies of Peripheral DP T cells

Total CD4CD8 DP T cells were quantified as percentage of total $CD3^+$ T cells in PBMC, and three subpopulations ($CD4^{hi}CD8^{lo}$, $CD4^{hi}CD8^{hi}$ and $CD4^{lo}CD8^{hi}$) according to the level of coreceptor expression could be distinguished as shown in figure 1A. $CD4^{hi}CD8^{lo}$ DP T cells are the most prominent of the DP cell populations and were found to be significantly more frequent in PBMC from ACPA positive RA patients when compared to healthy controls, SLE patients or ACPA negative RA patients (mean%: RA $ACPA^+$1.93, RA $ACPA^-$ 1.07, HD 1.24 and SLE 0.58, figure 1B). Total frequencies of CD4CD8 DP T cells were also higher in ACPA positive than in ACPA negative patients (figure 1C). The total CD4CD8 DP population will be used in all subsequent displays throughout the manuscript, unless otherwise stated. In the RA patient population no influence of age on the frequency of DP T cells was discernible (figure 1 D). Such a correlation was demonstrated for healthy individuals by others [18] and was also found in our own data for HD. (data not shown) A trend towards a further decrease of DP T cells in SLE patients compared to age matched controls did not reach statistical significance.

DP T cells in RA Belong to the Memory Pool of TCRαβ T cells and Display No Marker of iNKT cells

The previously described expansion of the memory pool in RA patients [12] was apparent in the analysis of CD4 SP T cells, 70% of which had a central memory phenotype characterized by expression of CD45RO and CCR7 (figure 2A). In agreement with previous reports [2,7], the majority of CD4CD8 DP T cells was also found to belong to the memory pool (87.9%). Analysis of CD45RO and CCR7 expression identified 58.4% as central memory cells (see figure 2A) and 29.5% as effector memory cells.

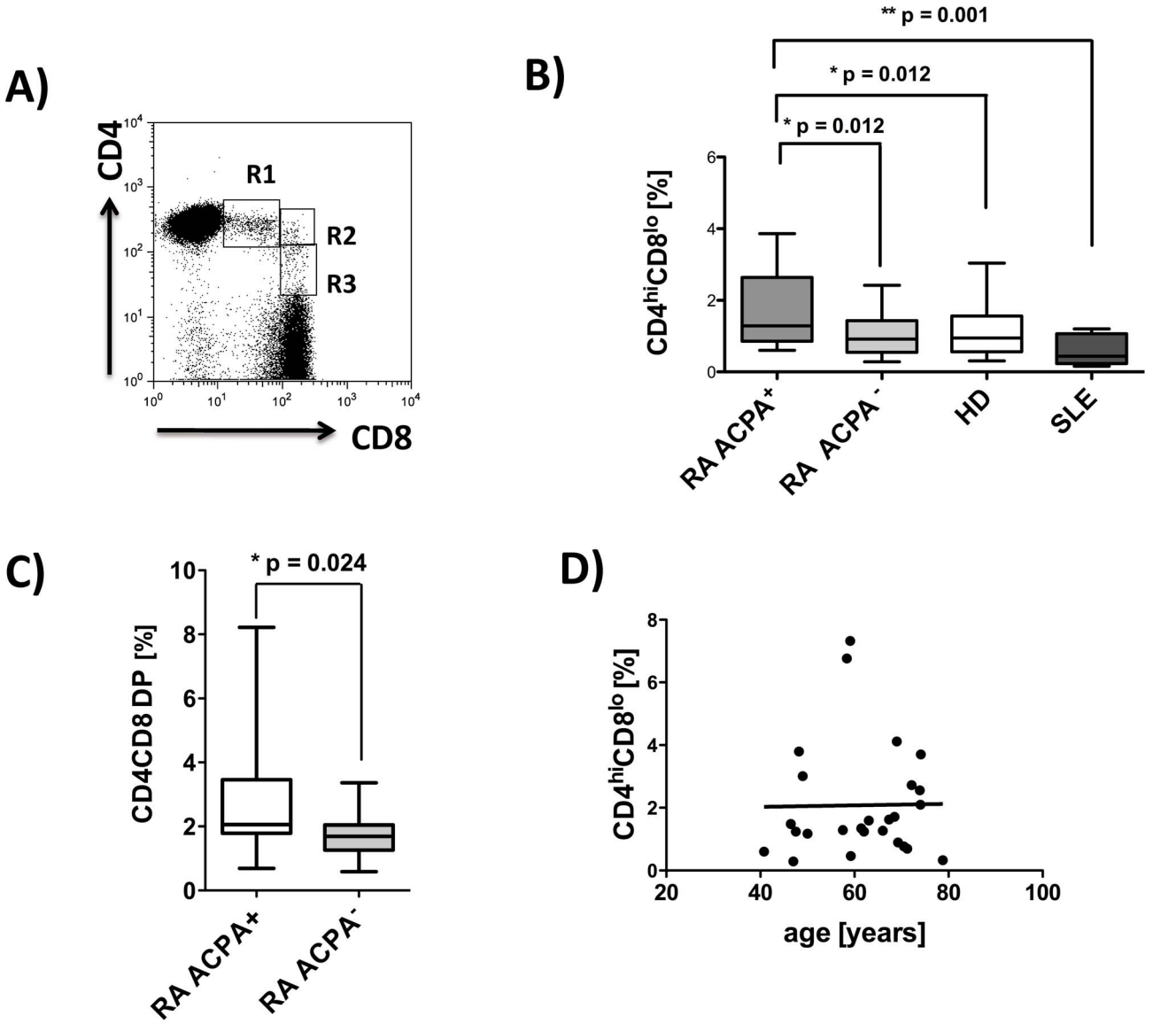

Figure 1. Increased frequencies of peripheral CD4CD8 DP T cells in ACPA$^+$ rheumatoid arthritis patients. PBMC were isolated from RA patients (ACPA$^+$, n = 37, ACPA$^-$, n = 20), healthy donors (n = 36), and patients with SLE (n = 8), and FACS analyses from live cells (PI staining used for exclusion of dead cells) were performed. Analyses for CD4CD8 double positive T cells are always pregated on CD3 positive T cells. **A)** Representative FACS plot from one RA patient, showing CD4CD8 double positive T cells, R1 = CD4^{hi}CD8^{lo}, R2 = CD4^{hi}CD8^{hi} and R3 = CD4^{lo}CD8^{hi} **B)** Comparison of frequencies of CD4^{hi}CD8^{lo}T cells in PBMCs of ACPA$^+$/− RA, HD and SLE patients. **C)** Total CD4CD8 DP T cells in ACPA$^+$ (n = 19) and ACPA$^-$ (n = 20) RA patients. **D)** Correlation analysis for frequencies of CD4^{hi}CD8^{lo} T cells with age in ACPA$^+$ RA patients. Box plots depict median, interquartile range and 10–90 percentile. Significance as given, *p<0.05, **p<0.01.
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The described spectrum of memory markers on DP T cells from RA patients did not differ from healthy controls (data not shown).

With regards to activation markers, DP T cells did not differ from CD4 and CD8 SP cells in their expression of CD38. Expression of the T cell activation marker HLA-DR [19], however, was found on DP T cells more frequently than on CD4 SP cells, but less frequently than on CD8$^+$ SP lymphocytes (figure 2B). In healthy donors, in contrast, HLA-DR expression on DP T cells did not differ from that of CD4 SP T cells (data not shown. CD56, which can also serve as activation marker for T cells [34], was also found significantly more frequently on DP T cells than on CD4 SP T cells (figure 2D). Other markers associated with activation/regulation like CD69 and CTLA-4 were not expressed on DP T cells (data not shown).

In peripheral blood from RA patients, CD4CD8 DP T cells expressed almost exclusively (99,3%) αβ T cell receptors, which was comparable to CD4 SP T cells (99,3%, figure 2 C). DP T cells were not γ∂ T cells (data not shown). Distribution of αβ and γ∂ TCR expression in the T cell subpopulations was similar in HD (data not shown). In accordance with previous reports [35], CD4^{hi}CD8^{lo} DP T cells in RA do not express αβ CD8 heterodimers but αα homodimers, whereas CD8 SP cells are almost exclusively CD8αβ positive (figure 2C).

DP T cells in HD have been described to partially belong to a population of T cells with NK phenotype, called NKT cells. [4]

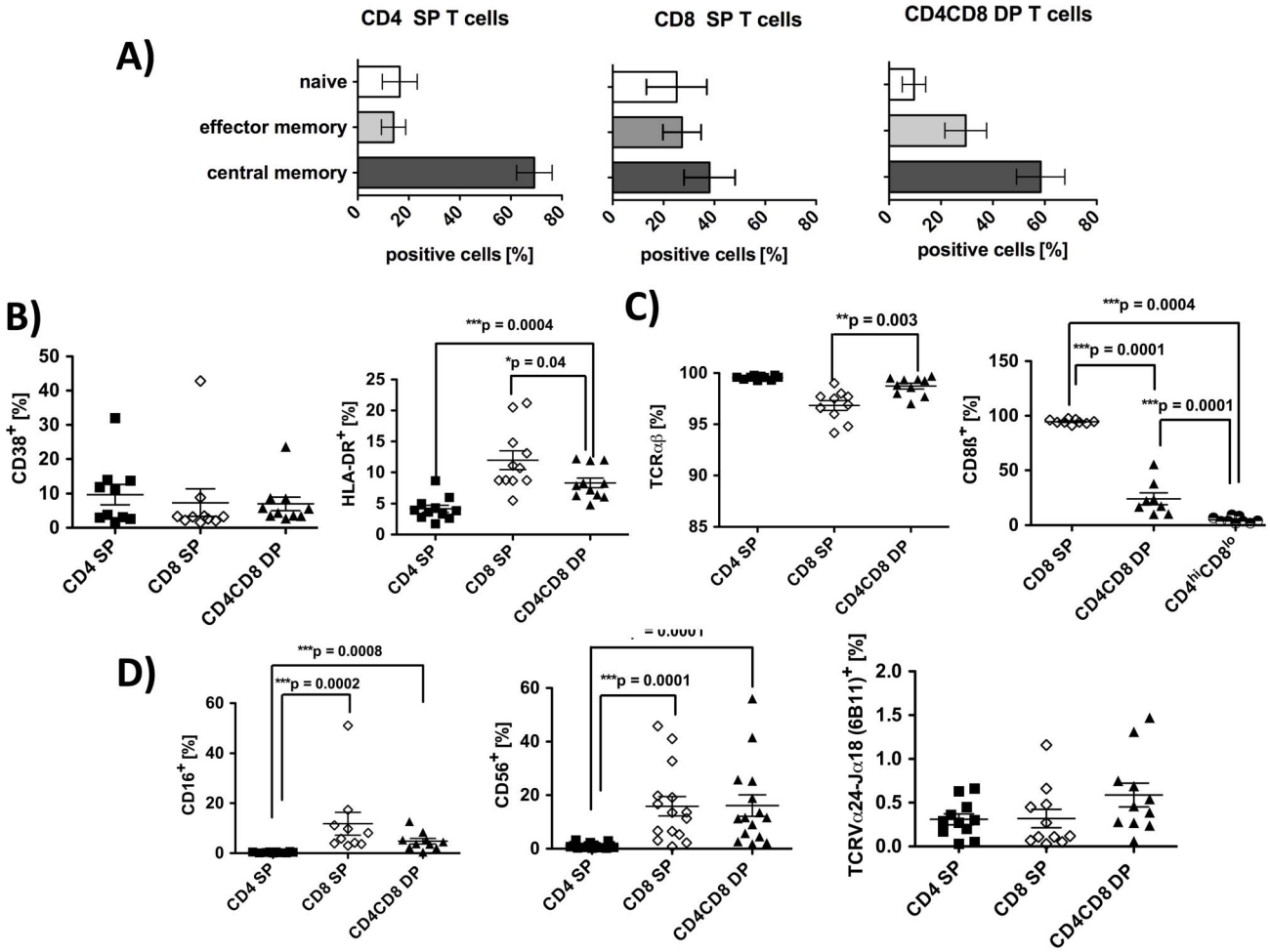

Figure 2. Phenotypic characterization of CD4CD8 DP T cells in RA patients. PBMC from RA patients were isolated and FACS analyses from live cells were performed (PI staining used for exclusion of dead cells). CD3 pregated cells were further gated on CD4 single, CD8 single and on CD4CD8 double positive T cells. **A)** Distribution of $CD45RO^{-}CCR7^{+}$ (naive) $CD45RO^{+}CCR7^{-}$ (effector memory) and $CD45RO^{+}CCR7^{+}$ (central memory) cells in the cell populations indicated. n = 5 5 **B)** Percentage of T cells positive for CD38 (n = 10) and HLA-DR (n = 11). **C)** Percentage of cells bearing a TCR alpha-beta (n = 10) and cells positive for CD8β (n = 9) in the T cell populations indicated. (**D)** Percentage of cells positive for CD16 (n = 10), CD56 (n = 15) and for TCRVα24-Jα18 (n = 11, marker for iNKT) in the T cell populations indicated. In all graphs, lines represent means and SEM. Significance as given, *p<0.05, **p<0.01, ***p<0.001.
doi:10.1371/journal.pone.0093293.g002

To determine a possible NKT cell phenotype of DP T cells in RA, we used an antibody recognizing the TCRVα24-Jα18:Vβ11, described for invariant iNKT cells, and a combination of anti-CD16 together with anti-CD56 to check for non-invariant NKT cells. The frequency of iNKT cells was not significantly increased in the DP fraction (0.59%±0.13) when compared to CD4 SP or CD8 SP cells (0.31% vs. 0.32%) (figure 2D). Frequencies of $CD16^{+}$ cells were low but were significantly increased in DP T cells (4.8%±1.1) when compared to CD4 SP cells (figure 2D). Co-staining of CD56 and CD16 on DP T cells revealed the presence of some infrequent (2.4%±0.47) $CD16^{+}CD56^{+}$ cells which possibly fall into the category of non-invariant NKT cells (data not shown).

To investigate the TCR repertoire used by DP T cells of RA patients, we performed FACS analysis using 24 different antibodies recognizing a broad range of TCR BV chains. The BV usage within DP T cells of 10 RA patients is divers and does not differ significantly from CD4 SP or CD8 SP. (figure 3A) 5 patients showed a substantial overrepresentation of individual BV elements (BV2, BV17 and/or BV21.3) (representative examples given in figure 3B), possibly indicating antigen-driven clonal expansion.

DP T cells are Present in the Synovial Tissue and Display Features of T helper Cells

The target organ in rheumatoid arthritis is the synovium. Therefore we were interested in identifying DP T cells in the synovium of inflamed joints. As demonstrated in figure 4A we do find CD4CD8 double positive T cells (pregated on $CD3^{+}$ T cells) in the synovium (mean: 2.5%). Of note, the same RA patients also showed $CD19^{+}$ B cells in the synovium (figure 3A).

To explore the potential of CD4CD8 DP T cells to provide B cell help, the CXCR5 expression was analyzed, since this chemokine receptor has been described to be present on several subpopulation of T follicular helper (Tfh) cells, although with varying support for antibody secretion [20]. The results showed that DP T cells from RA patients can express CXCR5, although the fraction of CXCR5 positive cells is higher in CD4 SP than in CD4CD8DP cells (mean: 9.5% vs. 4.1.%, p = 0.03, figure 4B).

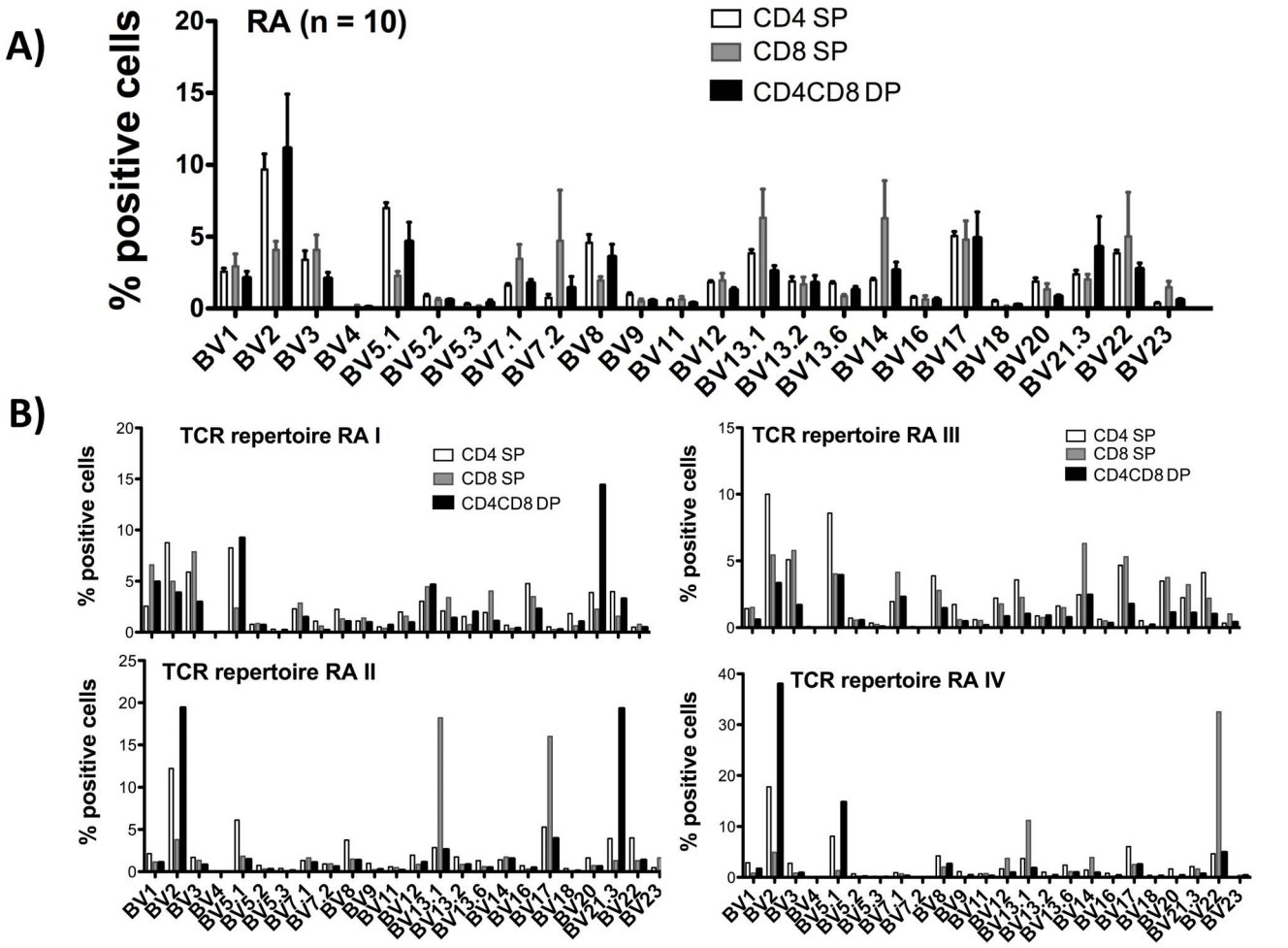

Figure 3. TCR BV distribution in CD4CD8 double positive T cells of RA patients. PBMC from RA patients were isolated and FACS analyses from live cells were performed (PI staining used for exclusion of dead cells). CD3 pre-gated cells were further gated on CD4 single, CD8 single and on CD4CD8 double positive T cells. Bar charts depict the percentage of cells positive for each BV element in the indicated T cell subpopulations investigated. **A)** Mean distribution of 24 TCR BV elements in peripheral blood T cells from 10 RA patients. **B)** Examples of the individual distribution of 24 TCR BV elements in 4 RA patients.
doi:10.1371/journal.pone.0093293.g003

Similar frequencies were found in DP T cells from healthy individuals (mean 5.1%, data not shown).

As functional and possibly more specific markers of Tfh cells, the ability to secrete IL-4 and IL-21 following restimulation in vitro was determined, since both cytokines are required for B cell help [20,21]. Determination of IL-4 secretion of DP T cells upon in vitro short term restimulation with Cytostim showed a substantial fraction of them to produce IL-4 (figure 4C). Importantly, this percentage of IL-4 producers was significantly higher in RA patients than in healthy controls (mean: 1.99% vs. 0.71%, $p = 0.0006$).

Most importantly, however, a large fraction of DP cells (15–19%) was found to produce IL-21, compared to only 2% of CD4 SP T cells (figure 4D). No difference between the IL-21 production of DP T cells from healthy donors compared to those from RA patients was detectable (figure 4D).

DP T cells in RA Preferentially Produce IFNγ but not IL-17

Several abnormal T cell subpopulations are pathogenetically relevant in RA, among them Th17 cells and IFNγ producers [22–24]. Using cytokine secretion assays as well as intracellular FACS analysis, IFNγ and IL17 expression was determined in vitro after short term restimulation with the MHC-TCR crosslinker Cytostim (IFNγ) or with PMA/Ionomycin (IL-17). Frequencies of cytokine producers were calculated as percentage of total CD4CD8 DP T cells, since cytokine expression was not restricted to a particular subpopulation ($CD4^{hi}CD8^{lo}$ or $CD4^{lo}CD8^{hi}$) of DP T cells.

In RA patients, significantly more DP T cells produced IFNγ than CD4 SP or CD8 SP T cells (mean 21.1% vs. 3.9% or 12.7%, $p = 0.0001$ and $p = 0.012$, respectively) (figure 5A). In HD, in contrast, there was no significant difference in the percentage of IFNγ producers between the 3 different T cell populations (CD4 or CD8 SP and DP T cells, data not shown). Most importantly, the frequency of IFNγ producing DP T cells in RA patients significantly exceeds the frequency found in HD (11.4%) (figure 5A).

Results for IL-17 were less conclusive. IL-17 was mainly produced by CD4 SP T cells, and not by CD8 SP or DP positive T cells (figure 5B). Nevertheless, a trend towards higher IL-17 production was seen in CD4 SP T cells from RA patients compared to CD4 SP from HD T cells (figure 5B).

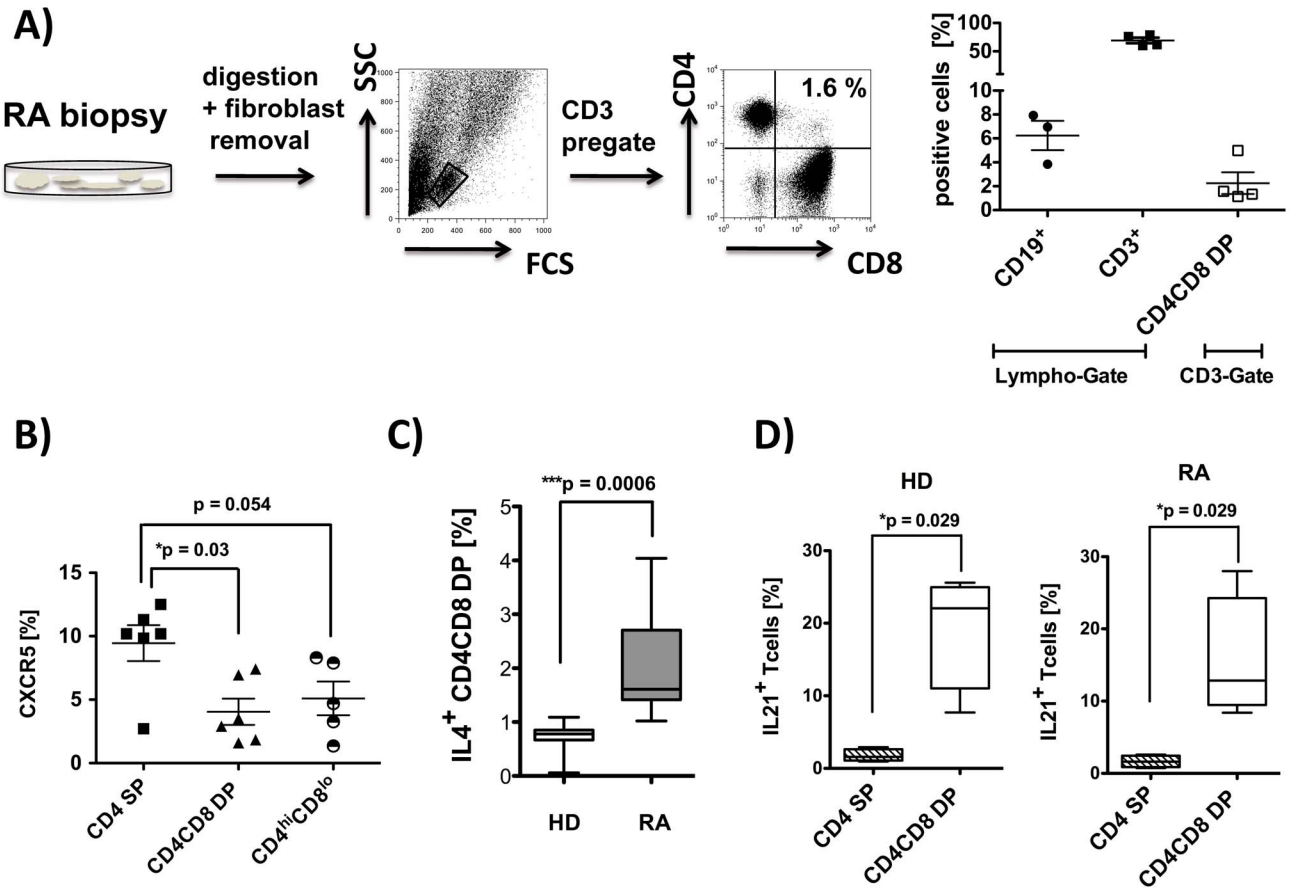

Figure 4. CD4CD8 double positive T cells are present in the synovium of RA patients and produce T helper 2 like cytokines. A) Single cell suspensions were prepared from synovial biopsies from RA patients (n = 4) and analyzed by flow cytometry using anti-CD19, anti-CD3, anti-CD4 and anti-CD8 monoclonal antibodies (PI staining used for exclusion of dead cells). Representative FACS plots are depicted. Each data point represents one experiment. **B)** PBMCs from RA patients (n = 6) were isolated and FACS analysis from live cells were performed (PI staining used for exclusion of dead cells). Cells were pre-gated on $CD3^+$ T cells, and CXCR5 expression was analyzed in the subpopulation indicated. **C)** PBMC were stimulated in vitro for 4 hrs with Cytostim. Subsequently, an IL-4 specific secretion assay was performed, and expression of CD4 and CD8 was determined in vital cells (PI staining used for exclusion of dead cells) pre-gated on CD3. Percentage of IL-4 producing CD4CD8 double positive T cells in HD (n = 7) and RA patients (n = 8) is given. **D)** PBMCs from HD (n = 4) and RA (n = 4) were stimulated with PMA/Iono for 4 hrs, and intracellular staining for IL-21 was performed. Cells are counterstained with anti-CD3, anti-CD4 and anti-CD8. Depicted is the percentage of cytokine producers in total CD4CD8 DP T cells and in CD4 SP cells. Box plots in C) and D) show median, interquartile range, and 10–90 percentiles. Significance as given, *$p<0.05$.
doi:10.1371/journal.pone.0093293.g004

DP T cells are more Frequent in Latent CMV Infection and Correlate with the Occurrence of CMV Specific Cytokine Producers

Latent CMV infection in rheumatoid arthritis is associated with a more severe cause of joint destruction, as published recently by our group and others [1,17]. This association was limited to ACPA positive RA. Based on the observed increase of DP T cells in ACPA positive RA, which are readily able to produce IFNγ in response to polyclonal stimuli, we decided to stratify the patients according to the presence or absence of anti-CMV IgG antibodies in the serum. The results showed significantly higher frequencies of $CD4^{hi}CD8^{lo}$ DP positive T cells, in anti-CMV IgG positive RA patients compared to CMV^- patients (mean 1.81% vs 0.71%, $p = 0.007$, figure 6A). This difference remained significant, if the analysis was restricted to ACPA positive patients only (mean 2.27% vs. 1.55%, $p = 0.039$). Interestingly, the highest frequencies of CD4CD8 DP T cells (mean%: 2.27) were found in patients simultaneously positive for anti-CMV IgG and ACPA (data not shown).

$CD4^+CD28^{null}$ T cells are increased in RA patients and associated with a more severe course of the disease [13,25]. In addition, they are known to occur in the course of a latent CMV infection both in RA patients and in healthy controls. Therefore, the frequency of $CD4^+CD28^{null}$T cells was determined in the RA patients and found to correlated with the percentages of total CD4CD8 DP T cells (figure 6B). This correlation was restricted to $ACPA^+$ RA patients and was not present in ACPA negative patients. Furthermore, DP T cells were found to preferentially have lost CD28 expression (mean%: 19,4, n = 13) in ACPA positive RA patients when compared to CD4 SP (mean%: 1.97) T cells, while the frequency of CD28 negative cells was even higher in CD8 SP (mean%: 33) T cells (figure 6B).

Finally, in order to investigate the CMV response, we restimulated (4 hrs) PBMC from anti-CMV IgG seropositive RA patients with CMV lysate or control lysate and determined the CMV specific IFNγ production of CD4 SP T cells and CD4CD8 DP T cells by cytokine secretion assay. The frequency of CMV specific IFNγ producers was found to correlate with the frequencies of total CD4CD8 DP T cells in CMV^+ RA patients

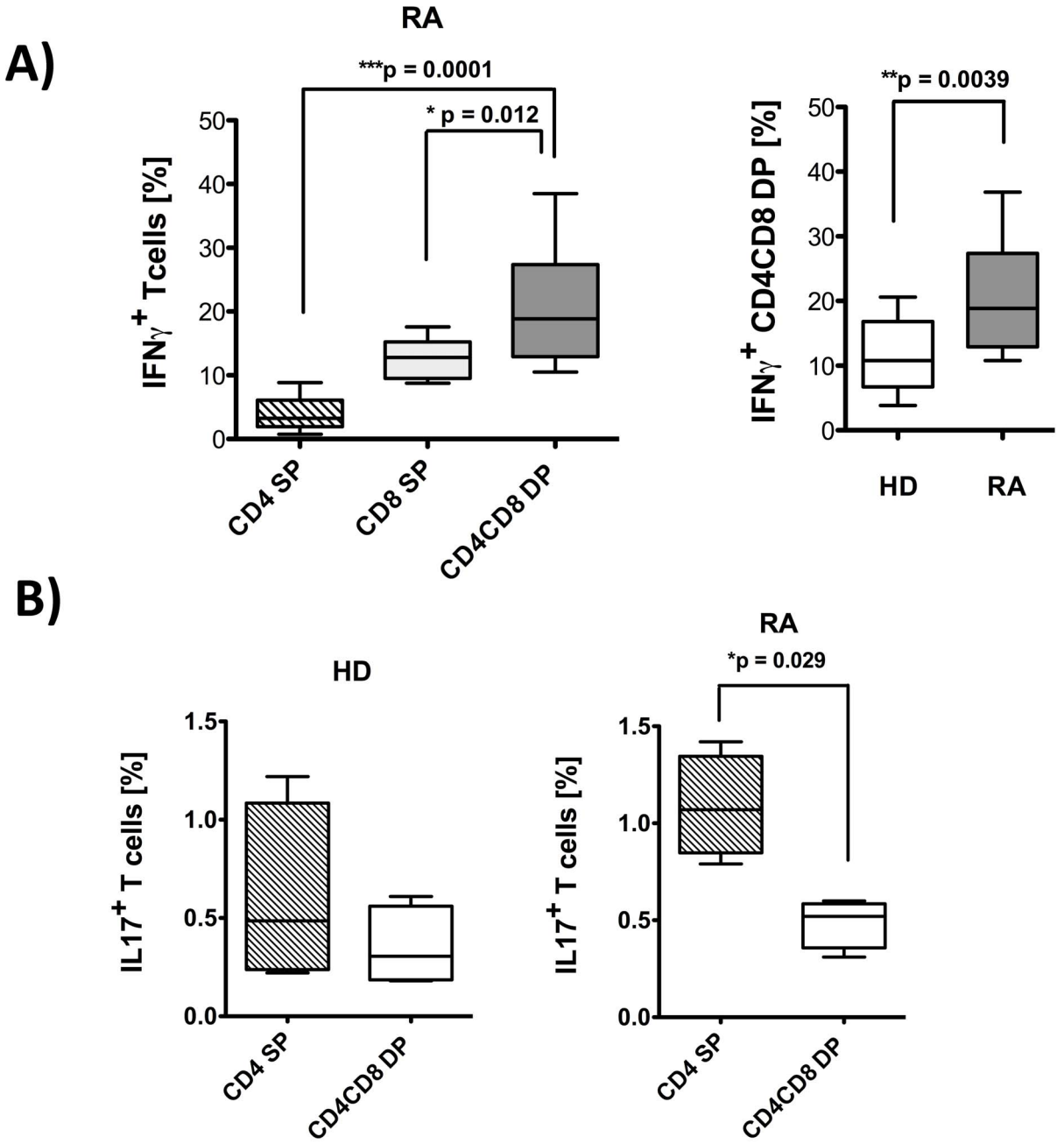

Figure 5. CD4CD8 double positive T cells from RA patients produce inflammatory IFNγ. PBMC from RA and HD were stimulated in vitro for 4 hrs with Cytostim (A) or PMA/Iono (B). Subsequently, cytokine specific secretion assays (A) or intracellular stainings (B) were performed and cells were counterstained with anti-CD3, anti-CD4 and anti-CD8. Flow cytometric cytokine analysis were performed on live cells for IFNγ secretion (PI staining used for exclusion of dead cells) or on fixed cells for intra-cellular staining for IL-17, in each case after pre-gating on $CD3^+$ T cells. **A)** Percentage of IFNγ producing T cells in samples from rheumatoid arthritis patients (n = 12, left panel and grey box in the right panel) and healthy donors (n = 13, clear box in the right panel) among CD4 SP, CD8 SP and CD4CD8 DP T cells. **B)** Percentage of IL-17 producing CD4 SP and CD4CD8 double positive T cells from RA patients (n = 4) and HD (n = 4). Presented box plots show median, interquartile range, and 10–90 percentiles. Significance as given, *p<0.05, **p<0.01, ***p<0.001.
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(figure 6C). Interestingly, the CMV specific IFNγ response of CD4CD8 DP T cells from RA patients was significantly higher (2.09) than in HD (0.67) (figure 6D). The antigen-induced, CMV specific proliferation of CD4 SP T cells, in contrast, did not correlate with the frequency of DP T cells (figure 6E).

Discussion

The study was conducted to investigate a possible role for peripheral CD4CD8 double positive T cells in the pathogenesis of rheumatoid arthritis.

The results show, that in RA patients, a significant number of mature T cells are indeed double positive for both co-receptors,

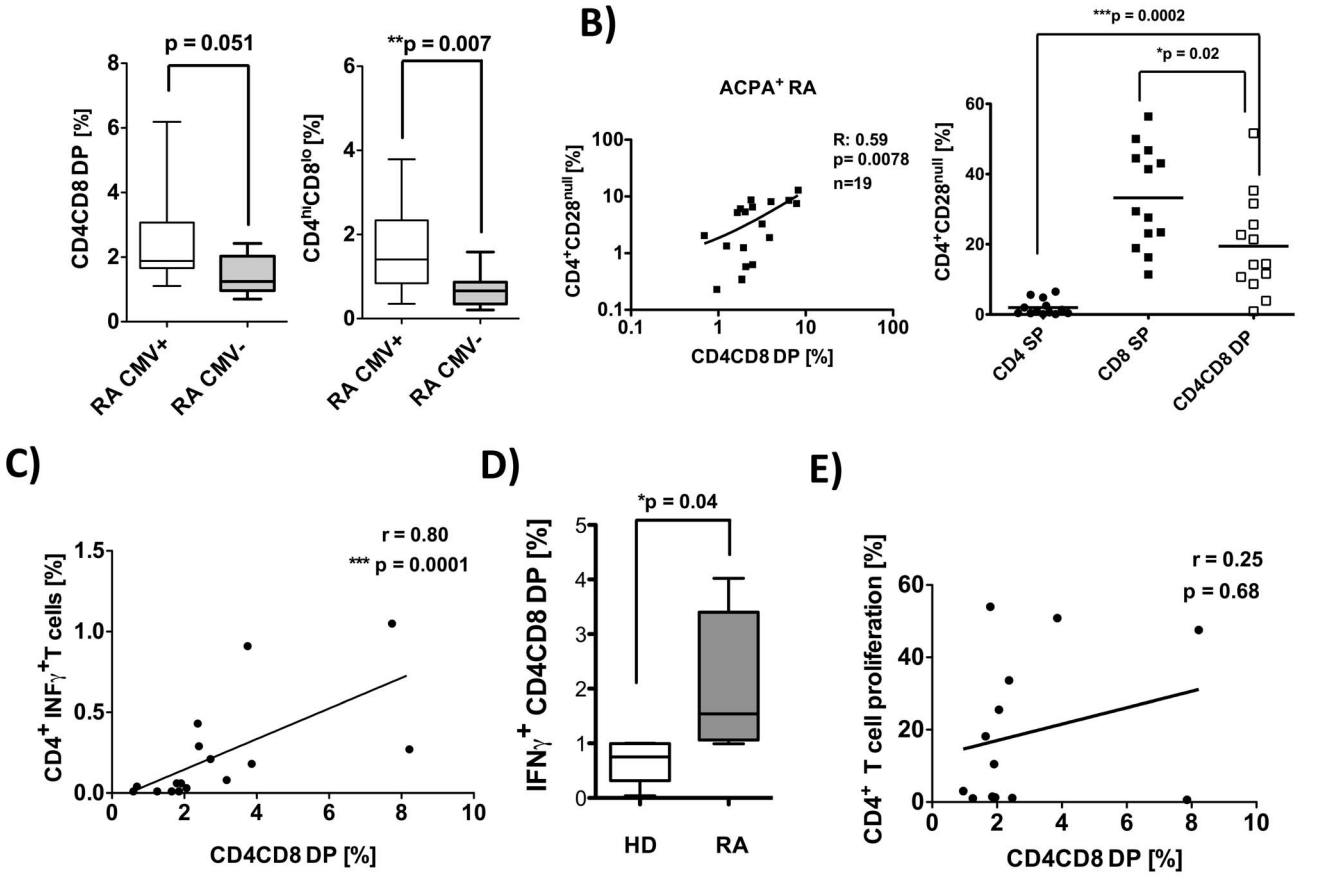

Figure 6. Frequencies of CD4CD8 double positive T cells are increased in CMV positive RA patients and correlate with CMV specific INFγ producers. A) PBMC were isolated from CMV^+ RA patients (n = 28) and CMV^- RA patients (n = 12) and analyzed by flow cytometry using anti-CD19, anti-CD3, anti-CD4 and anti-CD8 monoclonal antibodies (PI staining used for exclusion of dead cells). **B)** Correlation of the frequency of total double positive T cells with $CD4^+CD28^{null}$ T cells in $ACPA^+$ RA patients (left graph) and comparison of frequencies of $CD28^{null}$ cells among CD4 SP, CD8 SP and total DP T cells (right graph). **C–D)** PBMCs were restimulated with CMV-lysate for 4 hrs in vitro. Subsequently, an INFγ specific secretion assay was performed and cells were counterstained with aCD3, aCD4 and aCD8. Flow cytometric cytokine analyses were performed on live cells (PI staining used for exclusion of dead cells) pre-gated on $CD3^+$ T cells. **C)** Depicted is the **c**orrelation of CMV specific INFγ producing CD4 T cells with the frequencies of CD4CD8 DP cells from the same donor (n = 16). **D)** CMV specific INFγ producing CD4CD8 DP T cells from RA (n = 5) and HD (n = 5) are shown. **E)** PBMCs were labeled with CFDA-SE and restimulated with CMV-lysat (n = 13) for 7 d in vitro. Depicted is the **c**orrelation of CMV specific CD4 T cell proliferation with the frequencies of CD4CD8 DP cells from the same donor. Presented box plots show median, interquartile range, and 10–90 percentiles. Significance as given, *$p<0.05$, **$p<0.01$, ***$p<0.001$.
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CD4 and CD8. Those cells, which can also be found in healthy individuals – although in significantly lower numbers – preferentially display a memory phenotype, and have been reported to bear no markers of naïve T cells or recent thymic emigrants [3] which makes it unlikely that they escaped from the thymus as double positive cells.

Instead, their co-receptor expression pattern with higher CD4 than CD8 expression, and the fact that $CD4^{hi}CD8^{lo}$ are the most prominent DP T cell subpopulation, as well as the generally presumed pivotal role of CD4+ MHC class II restricted T lymphocytes in RA, suggests that those cells were originally CD4 SP T cells, which gained expression of the second co-receptor CD8. De novo CD8 expression in $CD4^+$ T cells under the influence of cytokines like IL-4 has already been described decades ago [26], but the paucity of IL-4 typically found in RA, makes such cytokine driven CD8 expression as an explanation for the emergence of DP T cells in RA less likely. Nevertheless, the increased IL-4 production of DP T cells in RA may sustain their CD8 expression in an autocrine manner.

Two new studies published very recently offer an alternative explanation, since they have identified defined transcription programs which can initiate CD8 gene expression in CD4 SP T cells, for example the silencing of the transcription factor Thpok [27,28] The precise mechanism underlying the emergence of DP T cells in RA remains to be determined, however.

Increased numbers of double positive T cells were detectable only in ACPA positive and not in ACPA negative RA patients. This suggests their involvement in the autoreactive T cell help provided for auto-reactive, antibody producing B cells in this disease. In line with this, a fraction of the double positive T cells was found to express the typical marker of T follicular helper cells, CXCR5. The percentage of CXCR5 positive cells amongst double-positive T cells was lower, however, than their percentage amongst CD4 SP T cells, which argues against a preferential role of DP T cells as TfH cells. Nevertheless, a proportion of DP T cells might provide B cell help, especially by their production of cytokines with a role in T-B cell collaboration, in particular of IL-21. The site for this B cell help could be non classical follicular

centers, which are found in the synovium of a sizable fraction of RA patients. [29,30].

RA patients with ACPA positive disease have a more severe course of the disease with extra-articular manifestations and increased subclinical arteriosclerosis compared to ACPA- patients [17]. The increased frequency of CD4CD8 DP T cells in our study in ACPA positive RA might reflect the more pronounced immune deviations in those patients and indicates that those cells might be involved in the destructive immune response in this disease.

Phenotypic characterization of DP T cells in RA reveals them to be αβ T cells of a memory phenotype. Similar characteristics have been published for DP T cells in healthy donors [3].

NK T cells have received increasing attention in autoimmunity research recently. DP T cells in RA are not invariant NKT cells, but a small fraction was positive for markers for non-invariant NKT cells. This is in contrast to published data [4] for HD and might indicate a pathogenetically relevant, disease specific role of DP T cell subpopulations in RA. Furthermore, TCR BV analysis reveals an overrepresentation of individual BV elements (BV2, BV17 and/or BV21.3) in DP T cells of some RA patients, possibly indicating antigen-driven clonal expansion.

DP T cells are present in the rheumatoid synovium. In addition, our results show that a high percentage of DP T cells have lost expression of the ubiquitous costimulatory molecule CD28, and that their frequency is increased in CMV^+ RA patients. $CD4^+CD28^{null}$ SP T cells are found in 25–70% of RA patients and are associated with a more severe course of the disease [13,25,31]. Recently, we reported CMV^+ RA to be associated with a more severe course of joint destruction [1], and latent CMV infection is known to lead to an expansion of $CD4^+CD28^{null}$ T cells both in RA patients and in healthy individuals.

The present study showed enhanced frequencies of DP T cells in CMV^+ RA. Recently, we reported an increased CMV specific IFNγ production in RA compared to CMV^+ healthy controls. [1] Furthermore, the frequency of DP T cells in the present study was found to correlate with the frequency of $CD4^+$ CMV specific IFNγ producers. Interestingly, a low but significant fraction of CD4 SP T cells, and even higher frequencies of the DP T cells in RA do produce CMV specific IFNγ, more frequently though than T cells from HD. This is in line with virus specific DP T cells described in HIV patients [7]. Taken together, those results suggests that DP T cells are at least in part the results of the same RA specific, CMV triggered perturbation in the peripheral T cell pool, which also leads to the occurrence of $CD4^+CD28^{null}$ T cells. A CMV specific enhanced effector response for DP T cells has already been found in HD [8] and DP T cells are therefore likely also to be involved in the enhanced CMV response in RA.

Independently of CMV specificity, we found DP T cells to produce high amounts of IFNγ, but no IL-17, upon polyclonal stimulation. IFNγ is a key cytokine driving the autoimmune response in RA [23,32]. DP T cells in healthy, aging individuals are known to be capable of producing IFNγ upon polyclonal restimulation [33]. CD28 negative Th cells in RA have also been shown to secrete IFNγ upon autoreactive stimulation, and in line with this, the DP T cells in RA are also preferentially CD28 negative. Therefore, CD4CD8 DP T cells in RA seem to be part of the pathological T cell pool characterized by replicative exhaustion, aberrant IFNγ production and phenotypic alterations including the loss of the ubiquitous co-stimulatory molecule CD28. DP T cells might be the result of a differentiation program set in place in terminally differentiated memory Th cells, that is switched on under conditions associated with immunosenescence, including the memory inflation observed in latent CMV infection or the chronic autoimmune response in rheumatoid arthritis.

Conclusion

In summary, we are the first to demonstrate increased frequencies of peripheral CD4CD8 double positive T cells in ACPA positive RA.

DP T cells are present in the inflamed synovium. These cells display features of T helper cells by the production of IL-4 and IL-21. Furthermore, the production of IFNγ, loss of CD28 and the association with CMV positive RA imply a functional contribution to the pathogenesis of the disease.

Author Contributions

Conceived and designed the experiments: DQ UW. Performed the experiments: DQ KR. Analyzed the data: DQ KR. Contributed reagents/materials/analysis tools: RS CB. Wrote the paper: DQ UW.

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Adaptive immunity

Basic

Research Article

Autoimmune arthritis induces paired immunoglobulin-like receptor B expression on CD4$^+$ T cells from SKG mice

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The chronic, destructive autoimmune arthritis in SKG mice, which closely resembles human rheumatoid arthritis, is the result of self-reactive T cells escaping thymic deletion. Since the inhibitory receptor LIR-1 is up-regulated on auto-reactive T cells in human rheumatoid arthritis, the role of its murine ortholog PIR-B was investigated. Peripheral CD4$^+$ T cells from SKG mice were found to frequently express PIR-B, and this population produces more frequently IL-17 upon in vitro stimulation compared to PIR-B$^-$ cells. A much larger fraction of PIR-B$^+$ T cells, however, was found to secret no IL-17, but IFN-γ. With regards to the clinical course of the disease, high frequencies of PIR-B$^+$ CD4$^+$ T cells were found to be associated with a milder course of arthritis, suggesting that the net effect of PIR-B expression is suppression of autoreactive T cells. Our results indicate that overexpression of PIR-B on IL-17-producing SKG CD4$^+$ T cells might represent an effective counter-regulatory mechanism against the destructive potential of those cells. More importantly, a major population of PIR-B$^+$ T cells in SKG mice appears to play an inhibitory role by way of their IFN-γ production, since high frequencies of those cells ameliorate the disease.

Keywords: Arthritis · CD4$^+$ T cells · Inhibitory receptors · PIR-B · SKG

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Introduction

Rheumatoid arthritis (RA) is a chronic debilitating autoimmune disease primarily affecting small joints of hands and feet, which can be associated with systemic autoimmunity leading to major organ vasculitis. T lymphocytes are generally regarded as important players in the autoimmune process, primarily due to the HLA class II associations of the disease.

Subsequent studies reported the emergence of oligoclonal T-cell expansions and multiple phenotypic aberrations in T cells from RA patients. We and others described increased frequencies of circulating CD4$^+$ CD28^{null} and of extra-thymic CD4$^+$ CD8$^+$ double-positive T cells in RA patients [1–6]. Altered T-cell signaling capacity—possibly due to chronic tumor necrosis factor (TNF) exposure—has also been described in RA [7, 8]. In addition, Th17-cell differentiation and function seems to be a major factor in RA pathogenesis [9–11].

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We previously reported in a clinical cohort of RA patients an increased frequency of $CD8^+$ T cells expressing LIR-1, an inhibitory receptor that has been associated with autoimmune thyroid disease [12] and multiple sclerosis [13]. To elucidate the role of LIR-1 positive lymphocytes in autoimmune arthritis, we chose the spontaneous murine SKG arthritis model due to its close resemblance to the human diseases RA. BALB/c ZAP-70^{W163C}-mutant (SKG) mice are characterized by a mutation in the gene encoding the Src homology 2 (SH2) domain of a component of the T-cell receptor signaling complex, zeta-chain-associated protein kinase 70, which causes chronic autoimmune arthritis in BALB/c mice [14, 15]. Altered T-cell signal transduction results in positive selection of otherwise negatively selected, i.e. deleted, auto-reactive T-cell clones. Subsequently, auto-reactive T cells enter the periphery and become pathogenic after activation. SKG mice spontaneously develop arthritis in a microbially conventional environment but not under specific pathogen-free (SPF) conditions [16]. Injection of zymosan, a yeast cell wall extract, induces activation of the innate immune system via toll-like receptor signaling and drives differentiation of pathogenic T cells, thereby triggering arthritis even under SPF conditions [16, 17].

Based on the structural and genomic homology analysis as well as on similarities in their inhibitory intracellular signal transduction pathways, the murine paired immunoglobulin-like receptor B (PIR-B) is considered to be the mouse ortholog of LIR-1.

PIR-B is expressed on B cells, mast cells, antigen presenting cells [18–20] or prethymic progenitors [21], but not on mature T cells or natural killer (NK) cells [19, 22]. It has been shown to bind both classical and nonclassical MHCI molecules on opposing cells (trans), but cis interactions of PIR-B - MHCI have also been described on mast cells or dendritic cells [23–25]. Both cis and trans interactions appear to induce constitutive tyrosine phosphorylation in PIR-B^+ cells [26, 27] and the resulting suppression by PIR-B may contribute to maintain their resting state. Furthermore, ectopic expression of PIR-B on peripheral T cells was shown to regulate T-cell activation [22]. These observations indicate an important role of PIR-B in cell inhibition and consequently immune tolerance [23].

Based on these findings, we sought to investigate the contribution of PIR-B expression in autoimmune arthritis model. We report here that in analogy to LIR-1 expression in the human disease RA, PIR-B is up-regulated on peripheral T lymphocytes in murine SKG arthritis. PIR-B-expressing $CD4^+$ T cells display an effector phenotype and contain not only pro-inflammatory Th17 cells, but also IFN-γ producers likely to exert an inhibitory effect in the chronic autoimmune process.

Results

PIR-B^+ $CD4^+$ T cells occur frequently in SKG mice and increase further with arthritis development

Based on our previous finding of increased LIR-1-expressing on T lymphocytes in human RA, and in particular in more severe disease, we investigated the expression of the LIR-1 ortholog PIR-B on T cells in the SKG arthritis model, which is regarded as the murine arthritis model with the closest resemblance to human RA.

In contrast to the tissue and cell type distribution patterns of PIR-B reported in the literature [18, 19], we found strong expression of PIR-B on a large fraction of $CD4^+$ T cells from arthritic SKG mice and on a low percentage of $CD4^+$ T cells from control mice of the BALB/c background strain (Fig. 1A). Expression of PIR-B mRNA in $CD4^+$ T cells was confirmed by RT-PCR (Supporting Information Fig. 1). The difference between the two mouse strains was present in $CD4^+$ T cells isolated from either spleen or lymph nodes. In the BALB/c background strain, no PIR-B^+ T cells were detectable in lymph nodes or peripheral blood, and only low frequencies in the spleen (Fig. 1A).

In SKG mice, the microbial environment is known to determine incidence and severity of arthritis, while increasing age is also an aggravating factor. This led us to investigate the influence of those factors on the frequency of PIR-B^+ in SKG $CD4^+$ T cells. Mice bred under non-SPF conditions, which develop a severe and progressive arthritis, were found to have significantly higher frequencies of PIR-B-expressing $CD4^+$ T cells than mice kept under microbially clean, germ free SPF housing (Fig. 1B). Nevertheless, SKG mice bred under SPF housing still had higher frequencies of those cells compared to control BALB/c mice (Fig. 1B). A marginal increase of frequencies of PIR-B^+ $CD4^+$ T cells in aged BALB/c mice kept under non-SPF conditions did not reach statistical significance (5.2 to 7.8%, $p = 0.42$, Fig. 1B).

Analysis of PIR-B expression in B cells, macrophages and dendritic cells revealed no significant differences between BALB/c and SKG mice (Fig. 1C), and no deviations from the frequencies reported previously [18] for other mouse strains. As expected, PIR-B was expressed on almost 100% of macrophages and dendritic cells with no differences between BALB/c and SKG mice (Fig. 1C).

PIR-B expression can be induced by T-cell receptor stimulation and indicates an activated phenotype

For phenotypic analysis and functional characterization of PIR-B^+ $CD4^+$ T-lymphocytes, splenic $CD4^+$ T cells from 6 month old SKG mice were used (Fig. 1D). Analysis of the phenotype of PIR-B^+ $CD4^+$ T cells from SKG mice showed that nearly 30% of them expressed CD25, with no significant differences between PIR-B^+ and PIR-B^- T cells (Fig. 1D). CD27 was found on the majority of $CD4^+$ T cells, but the frequencies of $CD27^+$ $CD4^+$ T cells were significantly decreased among PIR-B^+ compared to PIR-B^- $CD4^+$ T cells. PD-1 as a marker for ongoing inhibitory signaling and T-cell exhaustion was expressed on few cells, but the frequencies of PD-1^+ cells were nevertheless significantly increased on PIR-B^+ cells (Fig. 1D). CD69 was analyzed as a second activation marker and was found to be expressed more frequently on PIR-B^+ than on PIR-B^- $CD4^+$ T cells (Fig. 1D).

PIR-B expression on SKG $CD4^+$ T cells was inducible in vitro by incubation with CD3 antibody (Fig. 2A). Following in vitro stimulation of splenocytes with anti-CD3/ CD28, the resulting

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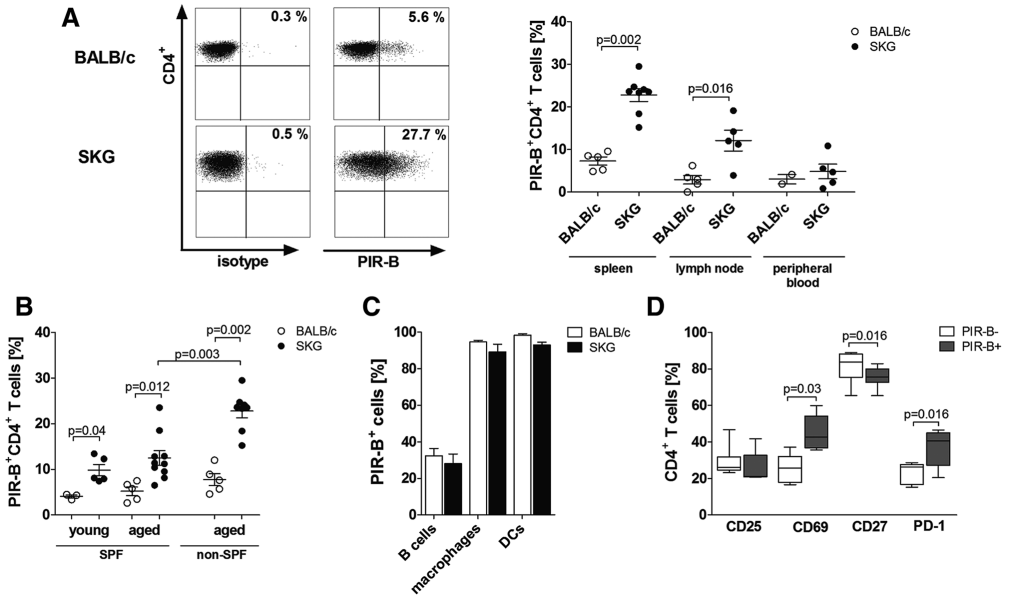

Figure 1. SKG mice show increased frequencies of PIR-B-expressing CD4$^+$ T cells with an activated phenotype. (A) Representative flow cytometry plots show surface expression of PIR-B on splenic CD4$^+$ T cells from aged BALB/c and SKG mice (40 weeks old, left). Depicted are frequencies of PIR-B$^+$ CD4$^+$ T cells in peripheral blood (n = 2 versus5), spleen (n = 5 versus8) and lymph nodes (n = 5 versus 5) from 40-week-old SKG mice with clinical signs of arthritis (right). (B) Frequencies of PIR-B$^+$ CD4$^+$ T cells from young and aged SKG (n = 5–11) and BALB/c (n = 3–6) mice, born and kept under SPF or non-SPF conditions. (C) PIR-B surface expression of B cells, macrophages and dendritic cells (DCs) isolated from spleens of BALB/c and SKG (n = 4) mice. (D) Frequencies of CD25$^+$, CD69$^+$, CD27$^+$ and PD-1$^+$ among PIR-B$^-$ and PIR-B$^+$ splenic CD4$^+$ T cells from 6 month old SKG mice (n = 7).
Circles represent individual mice; data in each graph are from single, independent experiments except graph B, where results from two independent experiments were pooled; horizontal lines indicate mean ± SEM. Box plot represents median (horizontal line), interquartile range (box) and minimum and maximum values. All frequencies are given as percent of total CD4$^+$ T-cell frequency unless indicated otherwise. Levels of significance as indicated. For statistical analysis Mann–Whitney test or Wilcoxon signed rank test was used.

frequencies of PIR-B$^+$ CD4$^+$ T cells were significantly higher in SKG than in the genetic background strain BALB/c (Fig. 2A and Supporting Information Fig. 2).

To test the functional response of T cells, their cytokine production was analyzed. Splenocytes from SKG and BALB/c mice were stimulated in vitro with anti-CD3/CD28 antibody, and IFN-γ secretion was determined. Following in vitro stimulation, BALB/c CD4$^+$ T cells were found to contain approximately 2% IFN-γ-secreting cells whereas frequencies of IFN-γ secretors were significantly lower among CD4$^+$ T cells from SKG mice (Fig. 2B). Nevertheless, in both SKG and BALB/c CD4$^+$ T cells, the frequencies of IFN-γ producers were significantly higher in PIR-B$^+$ compared to PIR-B$^-$ CD4$^+$ T cells (Fig. 2C).

In vitro activation with PMA/ionomycin leads to increased IFN-γ and IL-17 production in SKG T cells

Since T-cell receptor signaling is known to be attenuated in CD4$^+$ T cells from SKG mice, we used an alternative activation protocol with phorbol 12-myristate 13-acetate (PMA) and the Ca2+ ionophore ionomycin to bypass proximal TCR signaling. Divergent results from stimulation with anti-CD3 versus PMA/ionomycin have been interpreted as a possibility to distinguish impaired proximal TCR signaling due to the ZAP-70^{W163C}-mutation from signal transduction further downstream, which does not differ between SKG and BALB/c mice [28].

IFN-γ production of BALB/c CD4$^+$ T cells was stimulated by PMA/ionomycin in approximately 5% of the cells (Fig. 3A and B). The effect of PMA/ionomycin on CD4$^+$ T cells from SKG mice, however, was significantly different from the results seen with TCR ligation. The PKC mediated stimulation with PMA/ionomycin induced high frequencies of IFN-γ producers among CD4$^+$ T cells from SKG mice, which significantly exceeded the frequencies observed in BALB/c (Fig. 3A and B).

Since Th17-cells have been reported to be a pathogenetically relevant cell population in SKG arthritis [28] we also investigated the frequency of IL-17-producing CD4$^+$ T cells. Stimulation of splenic CD4$^+$ T cells with PMA/ionomycin was found to induce IL-17- production—determined by intracellular cytokine staining—in a subpopulation of CD4$^+$ T cells from SKG mice, but not from BALB/c mice (Fig. 3C).

Separate analysis of the PMA/ionomycin induced cytokine production of PIR-B$^+$ and PIR-B$^-$ CD4$^+$ T cells confirmed that approximately 5% of both PIR-B$^+$ and PIR-B$^-$ CD4$^+$ T cells from BALB/c mice produce IFN-γ with no significant difference between the two populations (Fig. 4A and B). As expected, neither population contained significant frequencies of IL-17-producers (Fig. 4A and C).

In SKG mice, in contrast, we found significant differences between PIR-B$^+$ and PIR-B$^-$CD4$^+$ T cells. While the frequencies

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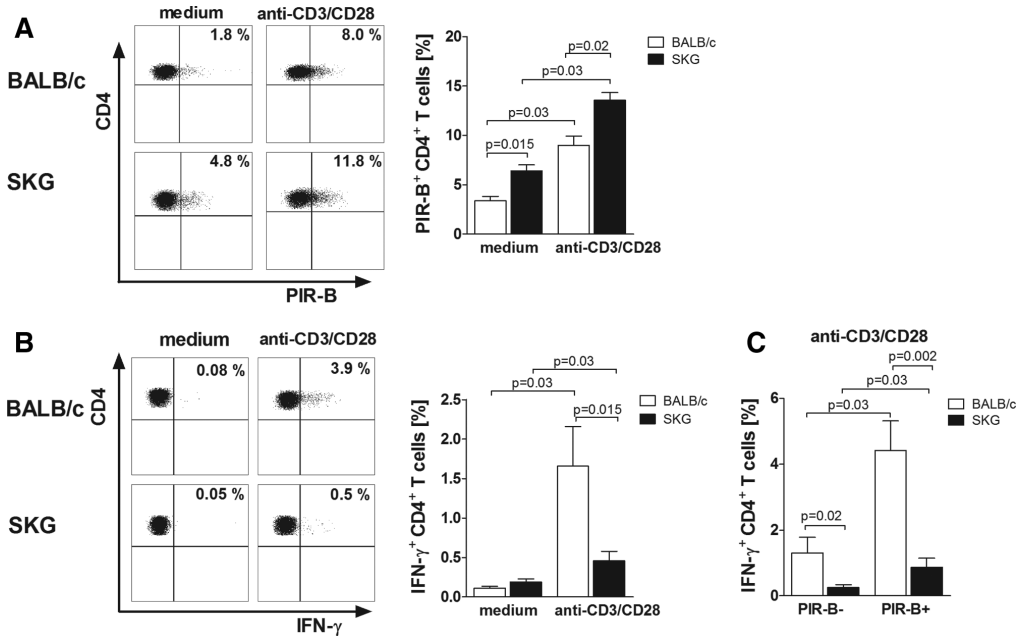

Figure 2. TCR-mediated activation induces PIR-B expression, but not IFN-γ secretion of splenic SKG CD4$^+$ T cells. (A) Representative dot plots and bar chart show PIR-B expression on CD4$^+$ T cells from BALB/c ($n = 6$) and SKG mice ($n = 6$) following in vitro stimulation with anti-CD3 (5 μg/mL) and anti-CD28 (2 μg/mL) versus controls. Data shown as mean ± SEM of PIR-B$^+$ CD4$^+$ T cells in the medium control (control) and after stimulation (anti-CD3/anti-CD28). (B) Representative dot plots and bar chart of IFN-γ secretion of CD4$^+$ T cells from BALB/c ($n = 6$ and SKG ($n = 6$) mice after in vitro stimulation with CD3/CD28 antibody for 4 h compared to the medium control. Bar chart depicts the frequency of IFN-γ-producing CD4$^+$ T cells (mean ± SEM) from BALB/c and SKG mice in the medium control and upon stimulation with anti-CD3/CD28 (right). Data from (B) and (C) frequency of IFNγ-producing CD4$^+$ T cells among PIR-B$^+$ and PIR-B$^-$ CD4$^+$ T cells ($n = 6$). Bar charts show results (mean ± SEM) from the indicated number of mice, which were pooled from three independent experiments. All frequencies are given as percent of total CD4$^+$ T-cell frequency unless indicated otherwise. For statistical analysis Mann–Whitney test or Wilcoxon signed rank test was used.

of IFN-γ producers were generally higher in SKG mice, we found the highest percentages of IFN-γ producers among PIR-B$^+$ SKG CD4$^+$ T-cell (Fig. 4D and E). The strong production of IL-17 in SKG CD4$^+$ T cells reported above was detectable in both PIR-B$^+$ and PIR-B$^-$ CD4$^+$ T cells. However, PIR-B$^+$ SKG CD4$^+$ T cells contained a significantly greater fraction of IL-17-producers than PIR-B$^-$ ones, and the frequencies of IL-17-producers among PIR-B$^+$ CD4$^+$ T cells were the highest observed in this study (Fig. 4D and F).

However, direct ex vivo analysis of PIR-B$^+$ and PIR-B$^-$ SKG CD4$^+$ T cells without in vitro stimulation revealed no detectable IL-17-secretion (data not shown).

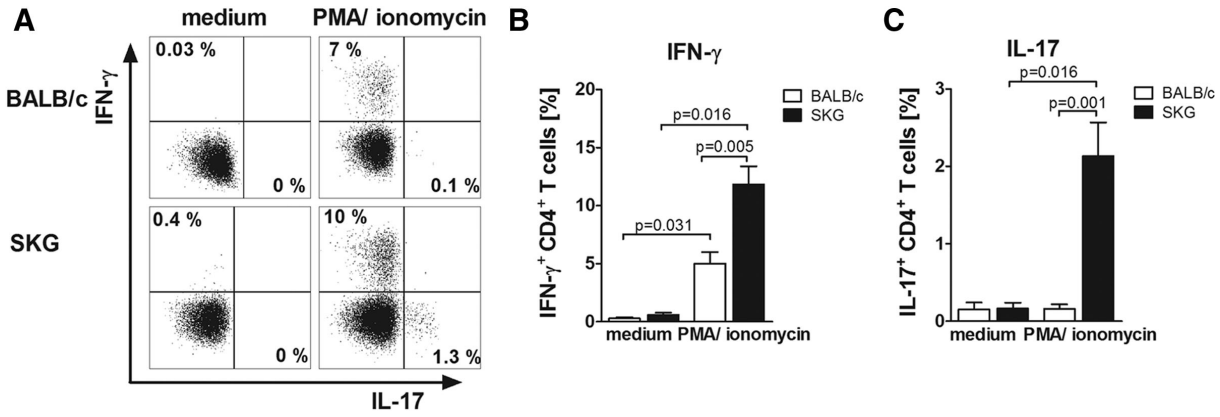

Figure 3. CD4$^+$ T cells from SKG produce high amounts of IFN-γ and IL-17. (A–C) Intracellular IFN-γ and IL-17 production of splenic CD4$^+$ T cells from 6 month old BALB/c ($n = 6$) or SKG mice ($n = 7$) was determined after 4 h stimulation with PMA (20 ng/mL) and ionomycin (1 μg/mL). Representative dot plots (A) and frequency of IFN-γ producers (B) and IL-17 producers (C) among stimulated CD4$^+$ T cells, compared to unstimulated medium controls (medium). All frequencies are given as percent of total CD4$^+$ T-cell frequency. Bar charts show results (mean ± SEM) from the indicated number of mice, which were pooled from three independent experiments. Levels of significance as indicated. For statistical analysis Mann–Whitney test or Wilcoxon signed rank test was used.

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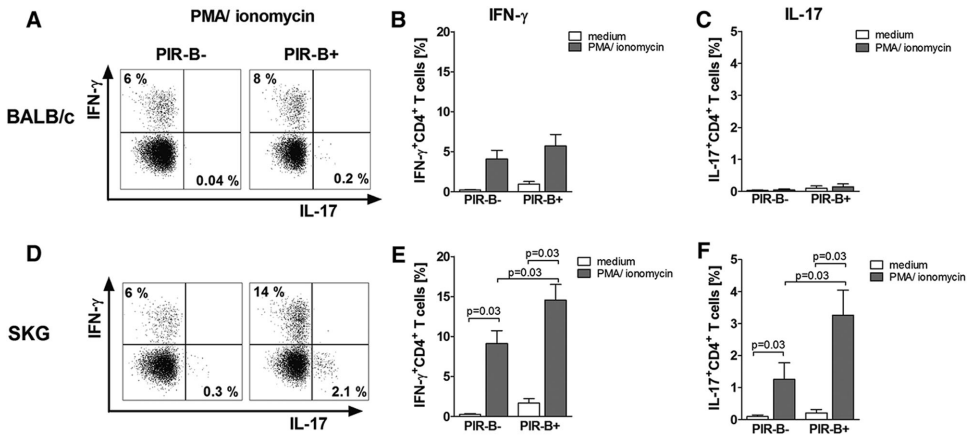

Figure 4. PIR-B$^+$ CD4$^+$ T cells from SKG mice are potent IL-17 producers. (A–C) Intracellular IFN-γ and IL-17 production of splenic PIR-B$^+$ and PIR-B$^-$ CD4$^+$ T cells from 6 month old BALB/c ($n = 6$) or SKG mice ($n = 7$) was determined after 4 h stimulation via PMA (20 ng/mL) and ionomycin (1 μg/mL). (B, D) Representative dot plots and frequency of (B) IFN-γ producers and (C) IL-17 producers among stimulated PIR-B$^+$ and PIR-B$^-$ BALB/c CD4$^+$ T cells, compared to unstimulated controls are shown. (D–F) Intracellular IFN-γ and IL-17 production of splenic CD4$^+$ T cells from SKG mice stimulated with PMA and ionomycin for 4 h. (D) Representative dot plots and (E, F) bar charts depicting the frequency of IFN-γ producers (E) and IL-17 producers (F) among stimulated PIR-B$^+$ and PIR-B$^-$ SKG CD4$^+$ T cells compared to controls. All frequencies are given as percent of total CD4$^+$ T-cell frequency. Bar charts show results (mean ± SEM) from the indicated number of mice, which were pooled from three independent experiments. Levels of significance as indicated. For statistical analysis Mann–Whitney test or Wilcoxon signed rank test was used.

IL-17-producing T cells are preformed while induced PIR-B$^+$ T cells are limited to IFN-γ production

In order to test the stability of the PIR-B phenotype in vitro, PIR-B$^+$ and PIR-B$^-$ CD4$^+$

T cells from SKG mice were preselected by cell sorter and incubated with and without PMA/ionomycin stimulation (Fig. 5A and B). The results show that PIR-B is up-regulated upon activation in approximately 10–15% of the previously PIR-B$^-$ CD4$^+$ T cells (Fig. 5C), while all PIR-B$^+$ T cells remained positive under the conditions described (data not shown).

When cytokine production was investigated, PMA/ionomycin stimulation was found to induce IFN-γ$^+$ cells among both PIR-B$^+$ and PIR-B$^-$ CD4$^+$ T cells (Fig. 5B). Again, the frequencies of IFN-γ producers were significantly higher in PIR-B$^+$ CD4$^+$ T cells, both in in vivo preformed and in vitro induced PIR-B$^+$ T cells (Fig. 5D).

IL-17 production, in contrast, was only found in the sorted PIR-B$^+$ CD4$^+$ T cells, which had already expressed PIR-B in vivo (Fig. 5B). In the ex vivo PIR-B$^-$ cell population, which in vitro had—upon stimulation with PMA/ionomycin—converted to PIR-B-positivity (Fig. 5C), only minimal IL-17-production was detectable, which was lower than in in vivo preformed PIR-B$^+$ cells (Fig. 5D).

To investigate mechanistically the influence of PIR-B signaling on IL-17-production, Th17-differentiation experiments were performed in vitro. For this, CD62L$^+$ naïve CD4$^+$ T cells were separated from BALB/c mice, since the ZAP70 mutation in the SKG strain prevents efficacious in vitro Th17-differentiation.

The results shown in Fig. 6A indicate that ligation of PIR-B with a specific monoclonal antibody did influence neither the frequency of emerging Th17-cells in those cultures (Fig. 6A) nor the expression level of IL-17 (data not shown).

Expansion of the PIR-B-expressing CD4$^+$ T-cell compartment is associated with lower arthritis scores

The observed preferential production of IL-17 in a small percentage of PIR-B$^+$ cells indicates a pro-inflammatory and tissue destructive effect of those cells. However, production of this pathogenetically relevant cytokine could be elicited by in vitro stimulation, but was not detectable in PIR-B$^+$ SKG CD4$^+$ T cells directly ex vivo (data not shown).

The strong bias towards IFN-γ production in a much larger fraction of PIR-B$^+$ cells, in contrast, indicates a potentially beneficial effect of those cells in SKG arthritis, since IFN-γ is known to inhibit Th17-differentiation in this disease [28]. This dichotomy prompted us to investigate the clinical consequences of increased PIR-B frequencies in the SKG mice.

The frequency of PIR-B$^+$ CD4$^+$ T cells in arthritic SKG mice determined ex vivo at 40 weeks of age was found to exert some influence on disease severity. When mice with PIR-B frequencies among CD4$^+$ T cells above 35% (cut off represents the median of determined frequencies of PIR-B$^+$ CD4$^+$ T cells) were compared to those with fewer PIR-B$^+$ cells, their arthritis score was lower with a significant difference at 32 weeks of age (Fig. 6B). In a separate experiment, the individual arthritis scores reached after 40 weeks showed a clear inverse correlation with the frequency of PIR-B$^+$ CD4$^+$ T cells determined (Fig. 6C). Histological analysis of the affected joints confirmed the association with disease severity with regards to immune cell infiltration and bone destruction. The histological scores reached at 40 weeks of age showed a significant inverse correlation with the frequency of PIR-B$^+$CD4$^+$ T cells (Fig. 6D and E).

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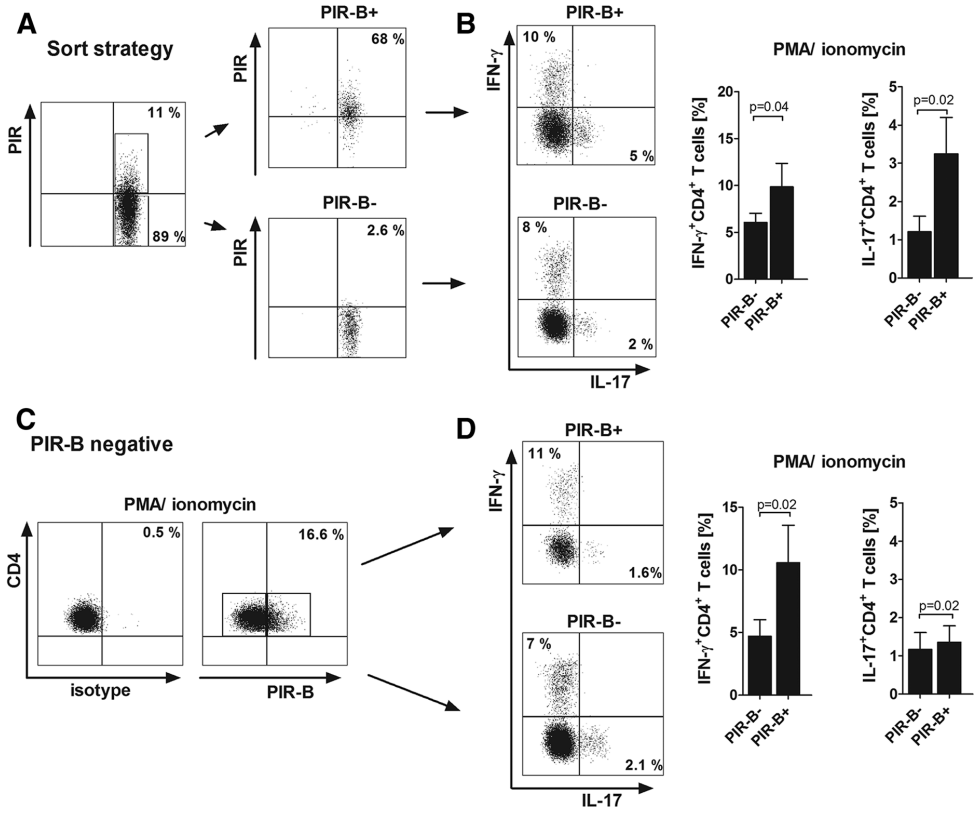

Figure 5. The PIR-B-expressing phenotype of SKG CD4$^+$ T cells is stable in vitro and is associated with IL-17 secretion. (A) Splenic CD4$^+$ T cells were separated and sorted for PIR-B$^+$ and PIR-B$^-$ T cells. Representative dot plots show sort strategy and re-analysis of sorted PIR-B$^+$ and PIR-B$^-$ CD4$^+$ T cells. (B) Representative dot plots show intracellular IFN-γ and IL-17 production in sorted PIR-B$^+$ (top) and PIR-B$^-$ SKG CD4$^+$ T cells (bottom) after stimulation with PMA (20 ng/mL) and ionomycin (1 μg/mL) for 3 h. The frequency of IFN-γ (left) and IL-17 producers (right) in PIR-B$^+$ and PIR-B$^-$ SKG CD4$^+$ T cells ($n = 8$) is shown. (C) Representative dot plots show PMA/ionomycin induced surface PIR-B expression and intracellular IFN-γ and IL-17 production after stimulation with PMA/ionomycin. (D) Separate analysis of previously PIR-B$^-$ CD4$^+$ T cells, which became positive (top) or remained negative (bottom). Representative dot plots show IFN-γ and IL-17 production of previously PIR-B$^-$ CD4$^+$ T cells after stimulation with PMA/ionomycin. Frequencies of IL-17$^+$ and IFN-γ$^+$ among PIR-B$^+$ and PIR-B$^-$ CD4$^+$ T cells from SKG mice ($n = 7$) are shown.
Bar charts show results (mean ± SEM) from the indicated number of mice, which were pooled from three independent experiments. Levels of significance as indicated. For statistical analysis Mann–Whitney test or Wilcoxon signed rank test was used.

Discussion

Previously, we and others have described an association of latent CMV–triggered immunosenescence with RA, in particular in patients with more severe disease [29–33]. LIR-1, an inhibitory receptor associated with T-cell compartment deviations characteristically induced in CMV infection, was found to be up-regulated on CD8$^+$ T cells from RA patients [30].This prompted us to investigate the expression of its murine ortholog PIR-B in an animal model of RA. We report here for the first time that PIR-B is up-regulated on peripheral CD4$^+$ T cells of SKG mice.

The chosen SKG arthritis is caused by a mutation in the gene for ZAP-70, which leads to attenuated TCR signaling [14]. The functional consequence of this mutation is thymic selection of auto-reactive T cells, which have attenuated TCR signaling, but react against self-antigens, [16]. We show here that a large fraction of the self-reactive CD4$^+$ T-cell pool in SKG mice is expressing PIR-B. PIR-B expression reached more than 25 % of CD4$^+$ T cells, in particular in aged mice, but was found only at low levels in the background strain BALB/c strain. This up-regulation occurs in arthritic mice and inducible to some extent in vitro, indicating that PIR-B$^+$ T cells are not selected in the SKG thymus due to the ZAP-70 mutation, but acquire this phenotype postthymically in the periphery as a consequence of chronic arthritis. Accordingly, PIR-B$^+$ CD4$^+$ T cells from arthritic SKG mice are activated and show decreased CD27 and increased PD-1-expression, indicating a preferential effector-memory differentiation status [34] with features of T-cell exhaustion. SKG mice have been suggested to be lymphopenic and, in analogy to human RA, be characterized by an increased homeostatic proliferation [35]. Accordingly, expansion of senescent and/or exhausted T-cell populations could contribute to increased PIR-B expression in SKG mice.

In SKG arthritis, CD4$^+$ T cells are commonly regarded as the most relevant cell population, and increased frequencies of PIR-B$^+$ CD4$^+$ T cells in arthritic mice are likely pathogenetically relevant [14]. Importantly, PIR-B$^+$ CD4$^+$ T cells are also more frequent in SKG mice housed under SPF conditions which

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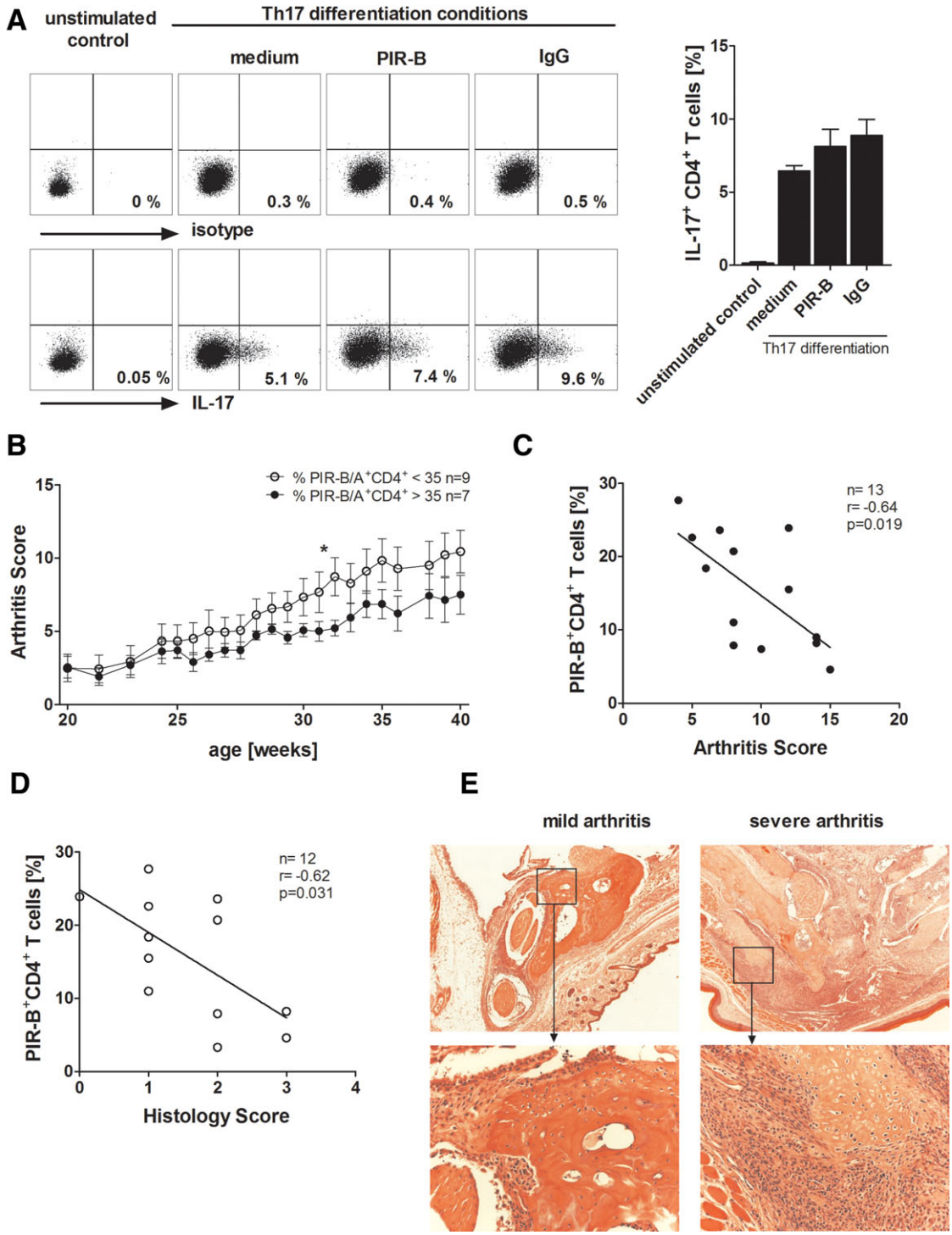

Figure 6. PIR-B ligation does not influence Th17-differentiation, and PIR-B expression ameliorates arthritis. (A) Splenic $CD4^+CD62L^+$ T cells from BALB/c mice were sorted and cultured without stimulation (unstimulated control) or under Th17-differentiating conditions in the presence of anti-PIR-B or control antibody (IgG). Incubation under Th17-differentiating conditions in medium alone was used as a negative control. After 3 d, cells were stimulated with PMA (20 ng/mL) and ionomycin (1 μg/mL) and stained for intracellular IL-17 production. Representative dot plots show intracellular IL-17 production of $CD4^+$ T cells under the indicated culture conditions of eight mice from one independent experiment. Horizontal lines indicate the mean ± SEM. Levels of significance as indicated. For statistical analysis Mann–Whitney test was used. (B) Clinical arthritis score was determined over 20 weeks of SKG mice ($n = 16$) bred under non-SPF conditions. After 40 weeks, frequencies of splenic $PIR\text{-}B^+$ $CD4^+$ T cells from SKG mice were determined and correlated with (C) arthritis score ($n = 13$) and (D) histology score ($n = 12$). Cut off represents the median of determined frequencies of $PIR\text{-}B^+$ $CD4^+$ T cells. Circles represent individual mice. Line illustrates linear regression. Levels of significance as indicated. For statistical analysis Spearman correlation test was used. (E) Histologic images representative for mild (score 1, left panel) or severe arthritis (score 3, right panel).

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do not develop arthritis. This could be a consequence of the involvement of $CD4^+$ T cells with attenuated TCR signaling in the early, antigen-specific events taking place due to the ZAP-70 mutation even before autoimmunity ensues, and might indicate an inhibitory effect of PIR-B expression already in this preclinical phase. Such an inhibitory function of PIR-B in chronic arthritis could subsequently be even more effective at sites of chronic inflammation. PIR-B has been reported to bind S100 A9 (also known as migration inhibitory factor-related protein 14 - MRP14- or calgranulin B) [36, 37], which is overexpressed in rheumatoid synovium and is found in increased concentration both in serum and synovial fluid of patients with RA [38, 39], while a decrease in S100A9 serum concentrations is associated with clinical improvement of the symptoms [40]. In addition, the human nonclassical MHC molecule HLA-G is also a ligand for LIR-1 and PIR-B [41] and treatment with soluble HLA-G produces excellent antiinflammatory effects in collagen-induced arthritis [42].

PIR-B has been shown to trigger an inhibitory signal in mast cells [25], eosinophils [43], macrophages [44, 45] and dendritic cells [46], but its role on T cells is only poorly understood. T cells of PIR-B deficient mice are characterized by increased IL-4 and decreased IFN-γ production [46], indicating a regulatory role in Th1/ Th2-balance. T or B-cell development is not altered in PIR-B deficient mice, and no overt autoimmune phenotype is detectable [22]. Ectopic expression of PIR-B on peripheral T cells, however, has been shown to trigger an inhibitory signal leading to suppression of proximal TCR signaling and attenuation of the IFNγ response [22].

In established, destructive SKG arthritis, IL-17 has been shown to be the pivotal cytokine, since the disease does not occur in IL-17-deficient SKG mice [28]. In our experiments, no IL-17 production was detectable in PIR-B^+ $CD4^+$ T cells, when analyzed directly ex vivo. This is in contrast to previous reports [28], but this discrepancy might be the result of the longer observation period of 40 weeks in our experiments compared to 12 weeks in previous studies. The frequency of PIR-B expressing cells increases with age, and the efficacy of PIR-B inhibition might result in the observed in vivo suppression of IL-17 production after 40 weeks.

The consequences of lymphocytic PIR-B expression for IFN-γ production are more complex, however. Imada et al. used thymocytes from a PIR-B-expressing transgenic mouse model with normal TCR signal transduction and saw a reduced IFN-γ response, which they interpreted as the result of cis-binding of PIR-B to MHC class I on the same cell surface. In contrast to this study, we saw an increase of IFN-γ secretion of ZAP-70 mutated SKG $CD4^+$ T cells expressing PIR-B, and PIR-B frequencies correlated with milder arthritis.

IFN-γ is known to ameliorate SKG arthritis due to suppressed expansion of Th17-cells in SKG mice, while an IFNγ knockout causes severe exacerbation [28]. IFNγ-deficient SKG mice develop arthritis even under SPF conditions, possibly indicating the suppressive effect of PIR-B^+ IFN-γ producers in SKG control mice. It seems feasible therefore, that the IFN-γ secretion of PIR-B^+ T cells contributes to the lower arthritis scores in mice with strong PIR-B expression. This hypothesis is further supported by our observation that IFN-γ production is increased in PIR-B^+ SKG $CD4^+$ T cells, underlining their potential role as "suppressors" of autoimmunity.

In contrast to PIR-B and IFN-γ expression, IL-17 production was not inducible in PIR-B^- cells by in vitro PMA/ionomycin stimulation, indicating that the IL-17 producers are preformed in vivo and are likely stable over longer periods of time. IL-17 differentiation also appears to be resistant to inhibitory PIR-B signaling, since PIR-B ligation did influence IL-17 differentiation in vitro.

In summary, a small fraction of PIR-B^+ $CD4^+$ T cells, which can be induced in vitro to produce IL-17, appears to be an auto-reactive population that is partially inhibited in vivo due to their PIR-B signaling. In addition, the results presented suggest that PIR-B^+, IFN-γ− producing $CD4^+$ T cells, which occur in much higher frequencies, are an efficacious inhibitory cell population in SKG arthritis. Those results support our previous findings of LIR-1 overexpression in human RA. If triggering of inhibitory signals via LIR-1 ligation results in increased IFN-γ production, than this might be a new therapeutic approach to RA or other autoimmune disease responsive to IL-17 inhibition.

Materials and methods

Mice

Female SKG and BALB/c mice were bred (BALB/c from the Medizinisch-Experiementelles Zentrum (MEZ) Leipzig or Jackson Laboratory) and maintained under SPF conditions or non-SPF conditions at the medical faculty of the University of Leipzig/MEZ. Animal studies were approved by the local regulatory agency (University of Leipzig).

Histopathologic assessment

SKG mouse right hind paws were fixed in formalin, decalcified, put in paraffin, cut and stained with H&E. The sections were scored at 40- and 200-fold magnification by two independent observers, for cellular infiltrations, exudations and pannus, bone erosions and cartilage destructions.

A semiquantitative graded scale from 0 to 3 was used as previously reported [47]: 0: no changes, 1: mild changes with low clinical signs of inflammation with cell infiltration, 2 moderate changes with cell infiltrations, low pannus and cartilage destructions and 3: severe changes with strong cell infiltration, pannus and cartilage destructions.

Scoring of joint swelling

Incidence and severity of arthritis were scored every week as described previously [14]. Scoring was performed as follows: 0: no joint swelling, 1: swelling of one finger joint, 2: at least

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two swollen finger joints, 3: mild swelling of wrist or ankle, 4: severe swelling of wrist and ankle. Scores of all forepaws and hind paws were totaled for each mouse.

Preparation of peripheral cells

Peripheral mononuclear cells were isolated from spleen and lymph nodes (iliac and inguinal) by disruption via cell strainer (70 μm; BD) and erythrocytes lysed with erythrocytes lysis buffer (CC Pro). After washing step with PBS, splenocytes were passed again through a cell strainer to remove cell aggregation. Peripheral blood cells were isolated by ficoll paque centrifugation.

Antibodies

The following antibodies were used: anti-CD3 (clone 145-2C11), anti-CD4 (clone: GK1.5), anti-CD19 (clone: 6D5), anti-PD-1 (clone: HA2-7B1), anti-CD11b (clone M1/70.15.11.5), anti-CD11c (clone: N418) (all from Miltenyi Biotec), anti-CD25 (BD Biosciences, clone: PC61), anti-CD27 (eBioscience, clone: LG.7F9), and anti-CD69 (eBioscience, clone: H1.2F3), anti-PIR-A/B (Biolegend, clone 6C1) and isotype control, PIR-B (R&D, clone 326414) and isotype control rat IgG2a (R&D), anti-IL-17 (Biolegend, clone: TC11-18H10.1) and anti- IFN-γ (Biolegend, clone: XMG1.2) and isotype control Rat IgG1 (Biolegend).

Cell staining and FACS

5×10^5 freshly isolated cells from blood, lymph nodes and spleen were centrifuged and incubated with conjugated antibodies for 10 min at 4°C, washed and analyzed by flow cytometry. Mouse Fc-blocking anti-CD16/CD32 (BD Biosciences) antibodies were used when PIR-A/B or PIR-B surface staining was performed. Dead cells were excluded from analysis by addition of propidium iodide or fixable viability dye (eBioscience).

When the study was initiated, commercially available antibodies specific for PIR-B could not discriminate between PIR-A and PIR-B. Accordingly, the PIR-A/B specific antibody (Biolegend, clone 6C1) was used. During the study, an antibody specific for PIR-B became available (R&D, clone 326414) and was tested on freshly isolated splenocytes. The results indicated, that the antibody specific for PIR-B stained slightly less cells than the PIR-A/B specific one, most likely due to expression of PIR-A on some PIR–B-expressing T cells. The PIR-B specific antibody was used for all experiments then.

Throughout the manuscript, the antibody used in the individual experiments is indicated specifically. Gating strategy is shown once in supporting information (Supporting Information Fig. 3).

For flow cytometry analysis, FACS Calibur and LSR II (BD Biosciences) and corresponding software applications CellQuest and Flowjo were used.

IFN-γ secretion assay

1×10^6 splenocytes were cultured in X-Vivo 15 medium and stimulated with 5 μg/mL plate-bound anti-CD3 and soluble anti-CD28 (2 μg/mL) antibody for 4 h. IFN-γ secretion assay (Miltenyi Biotec) was performed immediately afterwards. Then cells were stained for CD4, CD3 and PIR-B surface expression. Dead cells were excluded from analysis by addition of propidium iodide.

Cell sorting and Th17 differentiation

$CD4^+$ T cells from spleen of BALB/c wildtype mice were separated by magnetic cells sorting using $CD4^+$ T-cell isolation kit (Miltenyi) and stained in PBS with 1% FCS for 10 min at 4°C with anti-CD4, anti-CD3 and anti-CD62L antibodies (all from Miltenyi). Naïve $CD4^+CD62L^+$ T cells were sorted using a BD FACS ARIA sorter. For Th17 differentiation, sorted cells were activated by plate-bound anti-CD3 (eBioscience; 4 μg/mL) and anti-CD28 antibodies (eBioscience; 5 μg/mL) and cultured in X-Vivo 15 medium in the presence of the following cytokines: 2 ng/mL rhTGF-β, 20 ng/mL rmIL-6, 5 ng/mL rmIL-23; 10 μg/mL anti-IFN-γ and 10 μg/mL anti-IL-4-antibodies (all from eBioscience). Next, a monoclonal anti-PIR-B antibody or control antibody (both from R&D) was added to the cell culture. After 3 d, cells were stimulated with PMA (20 ng/mL) and ionomycin (1 μg/mL) for 4 h and stained for intracellular IL-17 production (as described below).

Intracellular cytokine production

For analysis of intracellular cytokine production, $CD4^+$ T cells from spleen were isolated by magnetic cell separation (MACS, Miltenyi Biotec) and stimulated with 20 ng/mL PMA and 1 μg/mL ionomycin in the presence of Golgi-Stop (1 μg/mL, BD Biosciences) for 4 h. Intracellular staining of IFN-γ and IL-17 was performed using the Inside Staining Kit (Miltenyi Biotec) or BD Cytofix/Cytoperm (BD Biosciences). Dead cells were removed by staining with Fixable Viability Dye (eBioscience).

RNA isolation, reverse transcription (RT) and real-time PCR analysis

FACS-sorted cells were resuspended in Sigma TRI Reagent (Sigma, Taufkirchen, Germany) and stored at -80°C. After extraction with chloroform, RNA was precipitated with isopropanol, washed twice with 70% ethanol and resuspended in DEPC water (all: Roth, Karlsruhe, Germany). In order to eliminate genomic DNA, RNA was treated with RNase-free DNase I (2,5 units per sample; Thermo Fisher Scientific GmbH, Germany) in the presence of RiboLock RNase inhibitor (20 units per sample; Thermo Fisher Scientific GmbH, Germany). Subsequently, RNA transcription to complementary DNA (cDNA) with High capacity cDNA Reverse Transcription Kit (Applied Biosystems, Germany) using a blend of random/oligo (dT) 18 primer was performed according to manufacturer's

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instruction. RT-qPCR was processed in duplicates with the iTaq TM Universal SYBR® Green Supermix (BIO-RAD, Germany) in iCycler iQTM5 (BIO-RAD, Germany). Following primers were used for RT-qPCR: mm_hprt_for: 5′-GTTGGATACAGGCCAGACTTTGTTG-3′ and mm_hprt_rev: 5′-GATTCAACTTGCGCTCATCTTAGGC-3′; mm_PirB_for: 5′-AGGATGGAGTGGAGCTGAAC-3′ and mm_PirB_rev: 5′-TGATTGTTTGCTCCTTGGCC–3′. The relative quantification of the transcripts was done by the $2(-^{\Delta\Delta}Ct)$ method.

Statistical analysis

For statistical analysis, GraphPad Prism 5.0 software (GraphPad Software, La Jolla, CA, USA) was used. Prior to all comparisons, a normality test was performed. Differences in medians or means between groups of the same mice were analyzed by Wilcoxon signed rank test or the Mann–Whitney rank sum test for different animals. Correlations were evaluated using Pearson's product-moment correlation or Spearman's rank correlation coefficient.

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Abbreviations: **IFN-γ**: Interferon gamma · **IL-17**: interleukin 17 · **PIR-B**: Paired immunoglobulin-like receptor B · **PMA**: phorbol 12-myristate 13-acetate · **RA**: rheumatoid arthritis · **SKG**: SKG mouse strain (Sakaguchi) · **Th17**: T helper cell 17 · **TNF**: tumor necrosis factor

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Letter to the Editor

High constitutive B7-H3 expression on human keratinocytes supports T cell immunity

Optimal T cell responses require T cell receptor (TCR) activation and signaling mediated by co-receptors, which are often members of the B7 family (B7-1, B7-2, BB-1, B7-H1 = PD-L1, B7-DC, B7-H2, B7-H3 – B7-H7). They exert co-stimulatory and/or co-inhibitory effects and their balance determines the fate of T cell responses [1]. In melanoma a relatively high B7-H4 expression was detected, which was associated with a more aggressive tumor behavior, poorer survival and altered immune cell infiltration [2]. In addition, a strong expression of B7-H3 was found in melanoma cells, but also in keratinocytes of the basal layer of *peri*-tumoral epidermis, even in cases of benign melanocytic nevi [2]. Since the number of T cells in healthy skin exceed twice that of peripheral blood [3] and several studies demonstrated the collaborative partnership of $CD8^+$ T cells and keratinocyte in co-cultures [4], we focused on the role of B7-H3 on keratinocytes and its potential role in T cell immunity by co-culture assays. We addressed the question,

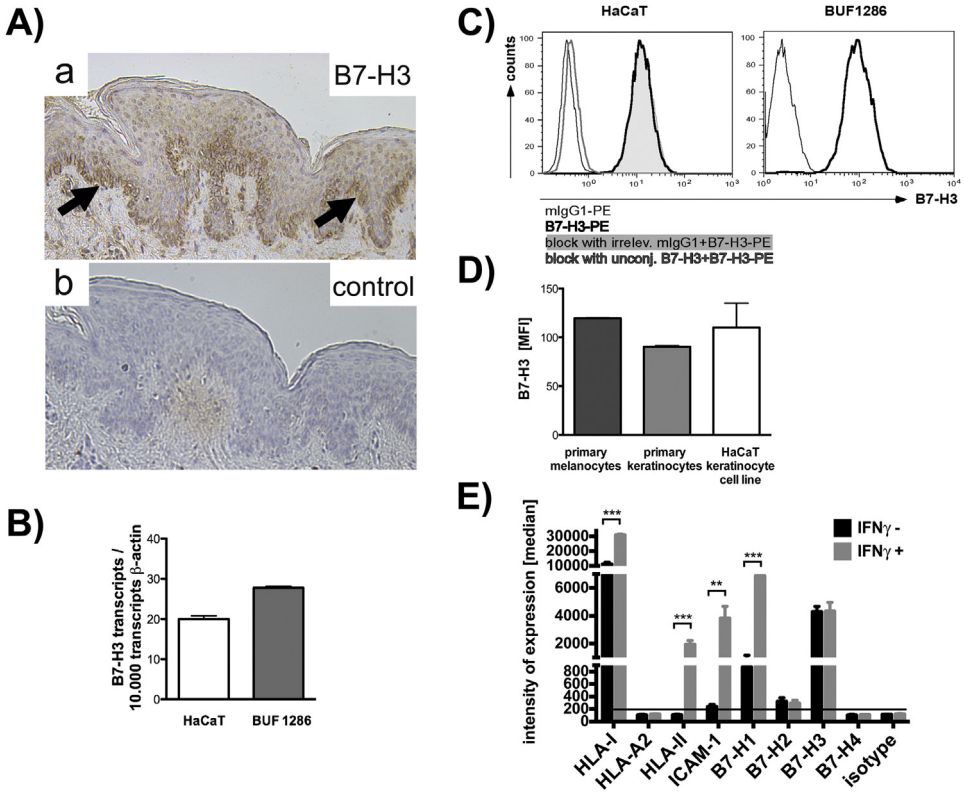

Fig. 1. Constitutively high expression levels of B7-H3 mRNA as well as protein in human skin and keratinocytic and melanocytic cells.
A) IHC was performed as described in Materials and Methods. Healthy human skin was stained with an anti-B7-H3 mAb (a), while a competition assay using recombinant mouse B7-H3 protein served as control (b).
B) Real time PCR data for the expression of B7-H3 mRNA on HaCaT and a representative melanoma cell line (BUF1286) are given. Using a B7-H3 plasmid as standard, B7-H3 transcript levels per 10.000 transcripts ß-actin are shown. Representative data from 3 independent experiments are depicted.
C) Surface B7-H3 (bold line) expression on HaCaT (left side) and a representative melanoma cell line (BUF1286, right side) as determined by flow cytometry are depicted. Thin line represents a respective isotype control. Blockade of the B7-H3 staining on HaCaT cells was included as specificity control. Representative histograms from at least 3 independent staining's are shown.
D) B7-H3 surface expression was determined by flow cytometry and depicted as mean fluorescence intensity (MFI) for primary human melanocytes, keratinocytes and keratinocyte cell line HaCaT. Combined data from 2 independent experiments are shown.
E) Surface expression of different immune relevant markers including B7-H molecules as determined by flow cytometry, depicted as median fluorescence intensity (median) for HaCaT cells +/– IFNγ treatment are given. Combined data from 3 independent experiments are shown. Significance as given $^{**}p < 0.01$, $^{***}p < 0.001$.

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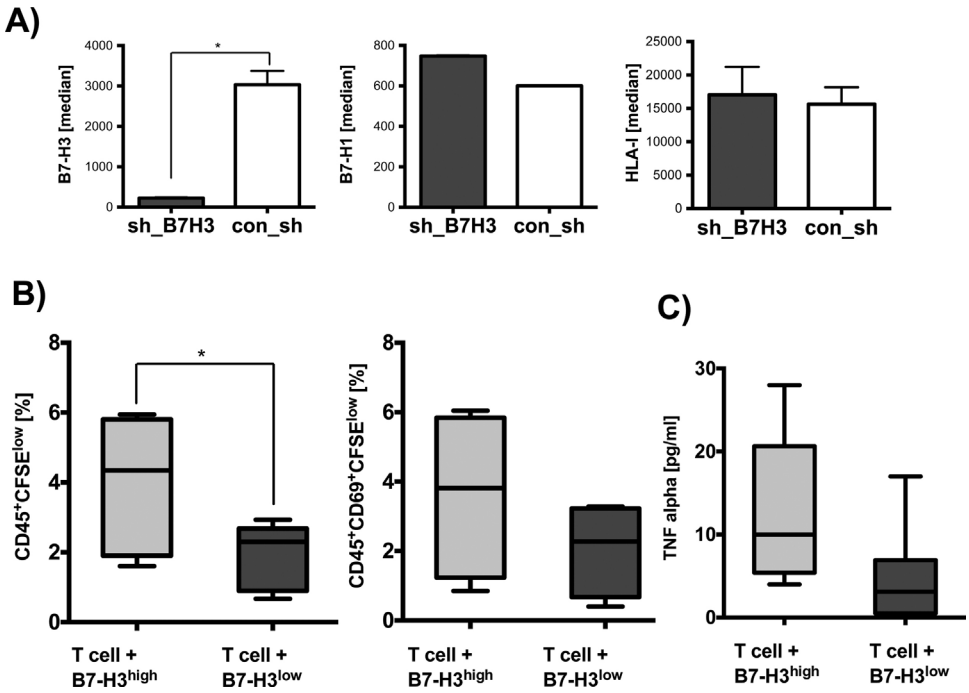

Fig. 2. B7-H3 on keratinocytes supports T cell immunity.
A) Inhibition of B7-H3 is shown by flow cytometry. HaCat cells were stable transfected with shRNA specific for B7-H3 (sh_B7H3) or control shRNA (con_sh) followed by subsequent FACS sorting for high or low B7-H3 expression. Cells were kept under G418 selection until use for co-culture experiments. B7-H1 and HLA class I expression were not affected by B7-H3 specific shRNA. Marker expression was independently analyzed 3 times. Significance as given $p < 0.05$
B + C) $CD8^+$ T cells were MACS sorted from PBMC (n = 6) and co-cultured for 5 days with 90 gy irradiated B7-$H3^{high}$ (control shRNA) or B7-$H3^{low}$ (B7-H3 shRNA) expressing HaCaT cells. Proliferation ($CD45^+CFSE^{low}$) and CD69 expression ($CD45^+CD69^+CFSE^{low}$) (B) as obtained by flow cytometry and TNFα secretion (C) as obtained by ELISA are shown. Significance as given $p < 0.05$.

whether the increased B7-H3 expression in basal keratinocytes is either a universal sign of human epidermis irrespective of the topographical region, or solely influenced by a primary skin tumor of benign or malignant nature.

While Youngnak-Piboonratanakit and co-authors [5] detected an expression of the inhibitory B7 family member B7-H1 on keratinocytes and could demonstrate an inhibitory role on T cell immunity in the inflamed skin, there exists so far no information about B7-H3 expression in keratinocytes. Therefore, the expression of B7-H3 was determined in human keratinocytes *in situ* by immunohistochemistry (IHC) of 67 different skin samples from 31 melanoma patients using an anti-B7-H3 monoclonal antibody (mAb). Staining with anti-B7-H3 mAb exhibited a high frequency of positivity (97.0% (65/67)) among all the samples analyzed. As representatively shown in Fig. 1a adjacent skin from benign lesions with 96.8% (30/31) positivity and adjacent skin from malignant lesions with 97.2% positivity (35/36) displayed both an intracellular and surface expression (score 2–3) of B7-H3 on keratinocytes *in situ*. The B7-H3 staining varied between different biopsies from lack of expression (score 0), weak (score 1), moderate (score 2) to strong (score 3) expression. The specificity of the B7-H3 staining was controlled as described in Materials and Methods and is depicted in (Fig. 1a, (b)).

With the exception of two skin samples obtained from the same patient that showed no B7-H3 staining, epidermal keratinocytes of all other samples expressed B7-H3 without any significant difference in the quality of B7-H3 expression in keratinocytes adjacent to either benign or malignant tumors (Supplementary Table 1). High mRNA expression levels of B7-H3 was found in healthy tissues of various organs, whereas protein expression was more restricted [6]. Although considerable numbers of tumors of distinct origin have been analyzed for the expression of B7-H family members [7], there is only little information available on B7-H3 expression and function in healthy human tissues and inflammatory skin diseases. In addition, high levels of B7-H3 mRNA (Fig. 1b) and surface protein expression (Fig. 1c-e) were detected *in vitro* on HaCaT cells, primary keratinocytes and melanocytes confirming the *in situ* data. In contrast to earlier published literature [8] HaCaT cells were HLA-A2 negative (Fig. 1e), but HLA-A31 positive as determined by genetic typing (data not shown).

To study the regulation of B7-H3, HaCaT cells were treated with IFNγ. When compared to untreated HaCaT cells, no change in B7-H3 expression was found upon treatment with this cytokine, whereas known immune markers like B7-H1, ICAM-1, HLA-I and HLA-II are highly upregulated by IFNγ exposure (Fig. 1e). A constitutive B7-H3 expression that was unaltered by inflammatory cytokines like TNFα, IFNγ, IL-1ß or IL-17, was previously shown for fibroblast-like synoviocytes and skin fibroblasts [9]. The precise function of the *trans*-membranous B7-H3 molecule is currently controversially discussed: While some studies described enhanced T cell effector functions in the presence of B7-H3 [6], the majority of reports assume rather an inhibitory effect of this molecule [10]. It is now well accepted that there exists both co-stimulatory as well co-inhibitory B7-H3 receptors on T cells, but the exact nature of the receptors is still under debate.

The function of B7-H3 on keratinocytes with respect to T cell immunity was tested by downregulating B7-H3 expression in HaCaT cells with B7-H3 shRNA (Fig. 2a). This caused a 12-fold reduction of B7-H3 surface expression, while the expression of other T cell immune markers like B7-H1 and HLA class I antigen expression remained unaltered (Fig. 2a). Co-cultivation of shB7-H3 transfectants with sorted $CD8^+$ T cells from HLA-A31 mismatched donors resulted in a reduced T cell proliferation, CD69 expression as well as TNF-α production when compared to co-cultivation with B7-$H3^{high}$-expressing HaCaT cells (Fig. 2b and c) suggesting a T cell

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supporting role of B7-H3 on keratinocytes. We found a rather low allo-specific T cell response upon keratinocyte co-culture with $CD8^+$ T cells from different donors. In other studies, low dose PHA was added to keratinocytes-T cell co-culture assays [5] or co-culture assays were performed with polyclonal activated T cells to boost otherwise low $CD4^+$ and $CD8^+$ T cell responses obtained with peripheral T cells *ex vivo* [4]. Addition of anti-CD3 to keratinocytes-T cell co-culture assays also boosted T cell responses (data not shown), whereas cultures with B7-$H3^{low}$ keratinocytes showed lower T cell effector functions in the presence of anti-CD3.

Taken together, our data suggest that a basal expression of B7-H3 on keratinocytes may contribute to a constitutive immunological function of human epidermis. Further experiments in autologous settings are needed to clarify whether B7-H3 indeed maintains T cell homeostasis and prevents overreaction to various and even minor exogenous or endogenous signals biologically negligible or even unwanted. Hence it seems most interesting to clarify whether basal keratinocytes, which are the first to meet epidermotropic lymphocytes, are pathogenetically involved in lymphocyte-mediated inflammatory epidermal disorders, like lichen planus, cutaneous variants of lupus erythematosus and graft-versus-host disease and even psoriasis vulgaris and allergic or irritant contact dermatitis.

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Conflict of interest statement

None to declare.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jdermsci.2017.02.287.

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Synergistic effects of IL-4 and TNFα on the induction of B7-H1 in renal cell carcinoma cells inhibiting allogeneic T cell proliferation

Dagmar Quandt, Simon Jasinski-Bergner, Ulrike Müller, Bianca Schulze and Barbara Seliger*

Abstract

Background: The importance of B7-H molecules for the T cell/tumor communication and its impact on renal cell carcinoma (RCC) progression and prognosis has been recently described. Cytokine treatment of RCC has earlier been shown to be beneficial in preclinical settings, but its clinical implementation has not proven to be as effective. This might be partially explained by the yet incomplete picture of cellular alterations in tumor cells upon cytokine treatment investigated in detail in this study.

Methods: RCC tumor cell lines were treated with different cytokines alone or in combination. The constitutive and/or cytokine-induced expression of cytokine receptors signaling components and B7-H molecules in RCC cells were analysed by qPCR and flow cytometry. A mcherry reporter gene construct containing B7-H1 promoter was cloned and its activity was determined upon transfection in cytokine-stimulated cells. Cytokine pretreated tumor cells were co-cultured with allogeneic $CD8^+$ T cells from healthy donors and T cell proliferation as well as cytokine secretion was determined.

Results: A heterogeneous, but constitutive B7-H1,-H2,-H3 and H4 expression was found on human RCC cell lines. IL-4 and TNFα treatment led to strong synergistic induction of B7-H1 in RCC cells, whereas B7-H2 was only increased by TNFα. In contrast, B7-H3 and B7-H4 expression were not altered by these cytokines. Treatment of RCC cells with TNFα and IL-4 was accompanied by an activation of signaling molecules like NF-κB, IκB and STAT6. The cytokine-mediated up-regulation of B7-H1 was due to transcriptional control as determined by an increased B7-H1 promoter activity in the presence of IL-4 and TNFα. Despite HLA class I and LFA-1 were also increased, the cytokine-mediated up-regulation of B7-H1 was more pronounced and caused an inhibition of allospecifc $CD8^+$ T cell proliferation.

Conclusion: Thus, IL-4 and TNFα, which could be released by immune cells of the tumor microenvironment, are able to control the B7-H1 expression in RCC thereby altering T cell responses. These data are of importance for understanding the complex interplay of tumor cells with immune cells orchestrated by a number of different soluble and membrane bound mediators and for the implementation of check point antibodies directed against B7-H1.

Keywords: Renal cell carcinoma, Costimulation, T cells, Anti-tumor response, B7-H molecules

Background

Renal cell carcinoma (RCC) is the most common kidney cancer type with an incidence of 5.8 in 100.000 people in the Western world [1] causing death of 5327 patients/year in Germany [2]. These results document the need for ongoing research to identify novel therapeutic strategies and to investigate mechanisms of tumor immune escape. RCC is considered an immunogenic tumor as demonstrated by a high frequency of tumor-infiltrating immune cells, a relatively high incidence of spontaneous recurrences as well as by the efficacy of immunotherapies, like DC-based vaccines, engineered autologous tumor cells, targeting T cell-tumor interaction, stem cell transplantation and treatment with cytokines [3].

B7 molecules are a growing protein family with diverse functions on both immune and tumor cells. They play in particular a key role in the crosstalk of the immune system and cancer tissues in different tumor entities [4]. B7 family members are mainly described to modulate T cell

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responses as second signal in cooperation with the first signal, the antigen recognition mediated by binding of the T cell receptor (TCR) with the major histocompatibility complex (MHC). These signals can be of co-inhibitory or co-stimulatory nature. Interestingly, also a reverse signaling in B7 family member expressing cells has been discovered [5]. The B7 family comprises B7-1 (CD80), B7-2 (CD86), B7-H1 (PD-L1), B7-DC (PD-L2), B7-H2 (ICOS-L), B7-H3, B7-H4 and B7-H6 [6,7]. Many tumors of distinct origin express B7-H molecules, in particular B7-H1. Therefore, monoclonal antibodies (mAb) targeting PDL1/B7-H1 on tumor cells or the PD1 receptor expressed by immune cells have been developed for the treatment of tumors. These antibodies are currently implemented in clinical trials demonstrating promising objective response rates in various tumors [8].

In the context of RCC B7-H1 expression of tumor lesions is associated with a worse prognosis of RCC patients [9]. The prognostic relevance of B7-H1 in RCC was further strengthened by the fact that B7-H1 mRNA is increased in early metastasis when compared to primary lesions suggesting that B7-H1 might serve as marker of a metastatic signature in RCC [10].

Cytokines are a family of modulatory proteins or glycoproteins that bind to their respective receptors on a variety of different immune and cancer cells thereby inducing different downstream signaling processes. Studies of cytokines are complicated due to their pleiotropy and apparent redundancies of action [11].

Over two decades one conventional treatment regime for patients with RCC included cytokines like IFN-α and IL-2. Despite the results were promising in preclinical settings [12-14], the clinical efficacy was rather poor with antitumoral responses ranging between 10 – 20% [15-18]. This might be due to the lack of knowledge of the tumor microenvironment, the molecular alterations and heterogeneity of tumors including those concerning the B7 family members in tumor and immune cells upon cytokine treatment.

With the exception of interferon (IFN)-γ [19,20] the effect of different cytokines on the regulation of B7-H molecules on RCC cells is widely unknown. Therefore, this study analyzed the regulation of B7-H molecules upon cytokine treatment in RCC in detail. B7-H1 surface expression was most dramatically altered upon IL-4 and TNFα. This enhancement occurred at the transcriptional level by direct upregulation of the B7-H1 promoter activity, which was associated with an inhibition of T cell proliferation.

Materials and methods

Cell lines and PBMC from healthy donors

The following RCC cell lines were used in the study and originally established from RCC patients in Mainz (MZ) or in Halle (Hal): MZ2514RC, MZ1257RC, MZ1790RC, MZ1774RC, MZ2733RC, MZ2877RC, Hal31RC, Hal162RC, Hal87RC and Hal149RC. The two melanoma cell lines BUF1088Mel and UKRV-Mel-14a have been recently described [21] and were either a kind gift from S. Ferrone (Pittsburgh, USA) or obtained from the European tumor cell line data base (ESTAB project; see www.ebi.ac.uk/ipd/estdab). Buffy coats were obtained from healthy donor (HD) of the blood bank of the University Hospital Halle. The Institutional Review Board (Ethics Committee) at the University Hospital in Halle (Germany) approved this study.

Reagents

Monoclonal antibodies (mAb) for flow cytometric analysis were: αCD8, αHLA-I (clone B9.12.1), αCD40 and αICAM-1 from Beckman Coulter (Krefeld, Germany); αPD1, αCD80, αNF-κB (pS529), αIκB and αpSTAT6 from Becton Dickinson (Heidelberg, Germany); αB7-H4 from AbD serotec; αICOS, αB7-H4, αB7-H1, αB7-H2 and αCD107a from ebioscience (Frankfurt, Germany); αB7-H3, αTNFRI and fluorokine biotinylated human IL-4 staining kit were used from R&D systems (Wiesbaden, Germany). Respective isotypes were purchased from BD Bioscience or Beckman Coulter, respectively. The antibodies were used unconjugated and/or as direct conjugates with FITC, Alexa-488, PE, APC or PE-Cy7. Recombinant TNFα and IL-4 for the treatment of RCC cell lines were purchased from ImmunoTools (Friesoythe, Germany).

IL-2 (Proleukine, Pharmacy, University of Halle, Germany), phorbol myristate acetate (PMA), propidiumiodid and ionomycin from Sigma-Aldrich (Steinheim, Germany) were used. αCD8 microbeads were obtained from Miltenyi Biotech (Gladbach, Germany). RPMI1640 and DMEM were purchased from Invitrogen (Karlsruhe, Germany), X-VIVO15 from Lonza (Basel, Switzerland). The fix and perm kit for intracellular stainings was from BD Bioscience.

The following antibodies for cell culture were employed: αCD3 (clone OKT3) and αB7-H1 (clone MIHI) from ebioscience, purified mIgG1 and mIgG2a from Millipore (Eschborn, Germany) and αHLA-I (clone w6/32) obtained from culture supernatants of hybridomas.

Flow cytometry

Flow cytometric analyses were essentially performed as recently described [21]. In brief, 1×10^5 cells were stained with fluorescent-labeled antibodies, while dead cells were excluded using PI staining. For determination of the IL-4 receptor expression an indirect staining method using IL-4-biotin followed by Avidin-FITC according to manufacturers' protocols was employed. For intracellular flow cytometric analyses of signal transduction components paraformaldehyde-fixed tumor cells were used, subsequently treated with permeabilizing buffer (methanol) prior to antibody staining. Flow cytometry was performed

using either a FACSscan™, FACSCalibur™ or a FACSCanto™ (all Becton Dickinson) or FC500 (Beckman Coulter) flow cytometer and CellQuest™ or CXP™ and FlowJo™ (Tree Star) software, respectively.

Cytokine treatment of tumor cells

3×10^5 tumor cells/well were seeded into 6 well plates in DMEM/10% FCS. Cytokines (IL-4 at 1000U/ml and TNFα at 800 U/ml) were added the following day for 30 min or 4–72 hrs as indicated for the subsequent analysis.

PCR analysis

Total cellular RNA from frozen cell pellets was extracted using RNAeasy MiniKit (Qiagen Hilden, Germany) and reversely transcribed into cDNA (Fermentas, St. Leon-Rot Germany) as recently described [22]. Semi-quantitative RT-PCR from cellular RNA was performed using the following oligonucleotide primers: For IL-4 *fw: 3′ cagttc tacagccaccatgaga 5′ rev: 3′ catgatcgtctttagcctttc 5′* for, IL-4Rα *fw: 3′ tctacttgcgagtggaagatga 5′ rev: 3′ ctccaaatgttgac tgcatagg 5′*, TNFα *fw: 3′ gtgcttgttcctcagcctct 5′ rev: 3′ gctt gtcactcgggttc 5′*, TNFRI *fw: 3′ gccaggagaaacagaacacc 5′ rev: 3′ gggataaaaggcaaagacca 5′* and for β-actin *fw: 3′ tcct gtggcatccacgaaact 5′ rev: 3′ gaagcatttgcggtggacgat 5′*. Real-time PCR (Cybr Green, Invitrogen) analysis for B7-H1 and B7-H4 from cellular RNA was performed using the following oligonucleotide primers: H1: fw: 3′ *gaactacctc tggcacatcct* 5′ rev: 3′ *gcccattccttcctcttgtc* 5′, H4: fw: 3′ agg cttctctgtgtgtctcttc 5′ rev: 3′ cttgctcttgtttgctcactcc 5′.

Cloning of the reporter gene vector

Genomic DNA was isolated from the B7-H1 expressing melanoma cell line UKRV-Mel-14a using the QIAamp DNA Mini Kit (Qiagen) according the manufacturers' protocol. The B7-H1 promoter was amplified by PCR with Taq DNA polymerase Kit (Invitrogen) employing the forward primer 5′-AAAGGTACCTAGAAGTTCAG CGCGGGATA-3′ and the reverse primer 5′-AAAGGAT CCCAGCGAGCTAGCCAGAGATA-3′. The specific PCR product was purified and cloned into the pMiR REPORT vector (Ambion, Austin, Texas, USA) using the restriction enzymes KpnI and BamHI (Fermentas) replacing the CMV promoter as recently described [23]. For replacing the luciferase (luc) reporter gene by the red fluorescent m-cherry protein, the m-cherry sequence was amplified from the pmR-m-cherry vector (Clontech, Mountain View, CA, USA) applying the forward primer 5′-AAAGGATC CATGGTGAGCAAGGGCGAGGA-3′ and the reverse primer 5′-AATGTGGTATGGCTGATTAT-3′. The PCR product was digested with BamHI (Fermentas) and SpeI (NEB, Ipswich, MA, USA) and cloned behind the B7-H1 promoter sequence in the pMiR REPORT backbone replacing the luciferase gene. The plasmid map is shown in Additional file 1: Figure S1.

Cell transfection

The reporter gene plasmid was stably transfected into the melanoma cell line BUF1088Mel using the Effectene Transfection Reagent (Qiagen, Hilden, Germany). Stable transfectants were selected with puromycin (pur) and a pur-resistant batch culture was generated. Transfected cells were cytokine treated as described above and flow cytometric analyses were performed 72 hrs post stimulation.

Tumor-T cell co-culture assays

Tumor cells were pretreated with cytokines as described above, detached, washed with PBS (3 x times), counted and seeded with $1 - 2 \times 10^5$ into 96 or 24 well plates. Peripheral blood mononuclear cells (PBMC) were obtained by Ficoll gradient from buffy coats of healthy volunteers. T cells were sorted for $CD8^+$ cells (purity > 98%) and co-cultivated tumor cells as described [21]. For proliferation assays, T cells were labeled with CDFA-SE (Lifetechnologies, Darmstadt, Germany) according to manufacturers' instructions) and tumor cells were pretreated with αHLA-I or anti-B7-H1 for 30 min prior to 5 day co-culture assays. Proliferation data are presented as division index (DI) that is the average number of cell divisions that a cell in the original population has undergone. For the determination of IFNγ secretion tumor cells were co-cultured with T cells for 4 hrs. Cell culture medium for the coculture assays was X-VIVO15.

Detection of cytokine release

To determine IFN-γ secretion of T cells the IFN-γ secretion assay (Miltenyi) was performed following the manufacturer's instructions. T cells stimulated with PMA/ionomycin (10 ng/ml and 1 μg/ml) served as a positive control. TNFα production of tumor cells was analyzed from culture supernatants using a TNFα-specific ELISA according to manufacturer's instructions (ebioscience).

Statistical analysis

Statistical analyses were performed using Prism 3.0 and depending on controlled data normality distribution Mann Whitney U Test or student's t test was used.

Results

Cytokine receptor expression as a prerequisite for cytokine activity in RCC cells

The constitutive expression of the IL-4 and TNFα receptors and and their ligands were determined in RCC cell lines. Using conventional qPCR TNFα, but not IL-4 mRNA expression levels were detected in the different RCC cell lines (Figure 1A). Despite prominent TNFα transcription rather low secretion levels of this cytokine were detected in one/five RCC cell lines tested (Figure 1B). In contrast, both the IL-4Rα and TNFRI were expressed

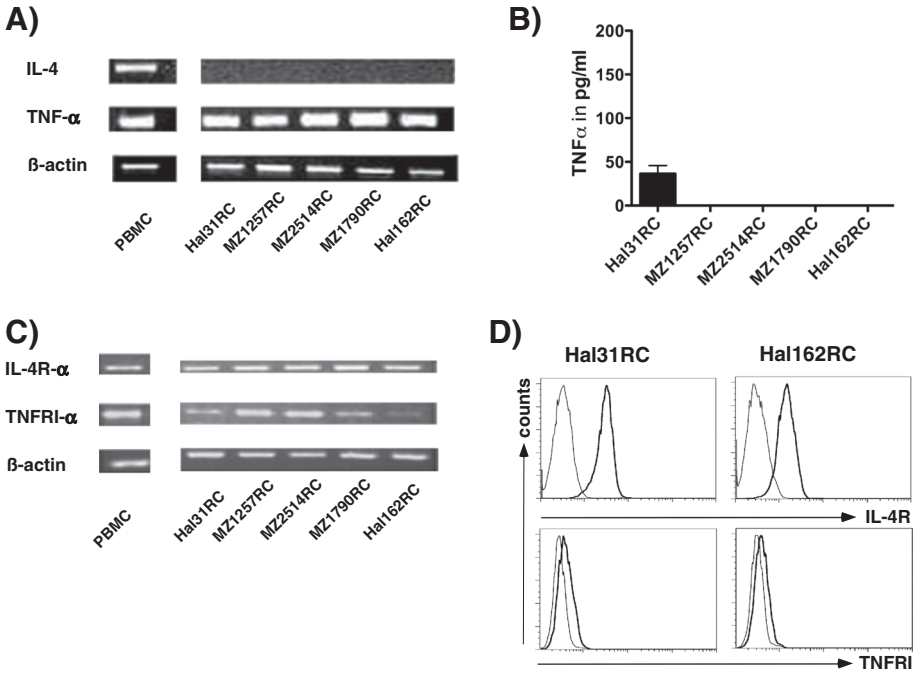

Figure 1 Expression analysis of IL-4, TNFα and their receptors in RCC cell lines. A) Constitutive TNFα mRNA expression, but lack of IL-4 mRNA expression was determined by conventional qPCR in different RCC cell lines (Hal31RC, MZ1257RC, MZ2514RC, MZ1790RC, Hal161RC) and in PBMC, which served as positive control. Representative data of at least 3 different experiments are shown. **B)** TNFα secretion of different RCC cell lines TNFα secretion was determined in Hal31RC, MZ1257RC, MZ2514RC, MZ1790RC, Hal161RC using ELISA. Representative data out of at least 3 different experiments are shown. **C)** The IL-4Rα and TNFRI-α expression was determined by qPCR in the different RCC cell lines and in PBMC, which served as positive control. Representative data of at least 3 different experiments are shown. **D)** Flow cytometric analyses of IL-4R and TNFαRI demonstrated the constitutive expression of IL-4R and TNFRI on Hal31RC and Hal161RC. The results are expressed as histograms. Bold line: staining, thin line: control. TNFRI is stained with an anti-TNFRI-PE labeled antibody, bold line: staining, thin line: isotype control. Representative data of at least 3 different experiments are shown.

at the mRNA (Figure 1C) and protein level (Figure 1D) as determined by qPCR and flow cytometry, respectively.

IL-4 and TNFα synergistically enhance B7-H1 expression in RCC cells

A heterogeneous, but constitutive cell surface protein expression of B7-H family members was detected in the tested RCC cell lines (Table 1). While B7-H1 to B7-H4 were expressed on all RCC cell lines, the degree of expression was quite diverse with the highest levels for B7-H3 followed by B7-H2 and B7-H1, whereas only weak, but detectable B7-H4 expression levels were found (Table 1). In addition, qPCR analysis revealed low B7-H4 mRNA levels (Additional file 2: Figure S2). 2/3 RCC lines were also weakly positive for B7-DC (data not shown). Upon treatment with either IL-4 and TNFα alone or in combination, the B7-H molecule expression was differentially affected: B7-H4 transcription (data not shown) and protein expression (Figure 2) was unaltered by this cytokine treatment. Despite high constitutive expression levels, a slight cytokine-mediated induction of B7-H3 was shown, which was most prominent in RCC31 upon IL-4 and TNFα treatment. B7-H2 was induced by TNFα, but not by IL-4 in the RCC cell line analyses, while combined TNFα with IL-4 treatment had no additional effects (Figure 2). The most prominent induction was found for B7-H1 in all RCC cell lines by treatment with either cytokine, while combination treatment caused synergistic effects

Table 1 Constitutive B7-H1-B7-H4 expression in renal cell carcinoma cell lines as determined by FACS, isotype control stainings were implemented

cell lines	B7-H1	B7-H2	B7-H3	B7-H4
Hal31RC	+	+	++	+/−
Hal78RC	+	+	++	+/−
Hal149RC	+	+	++	+/−
Hal162RC	++	++	++	+/−
MZ1774RC	+	+	+++	+/−
MZ1790RC	+/−	++	++	+/−
MZ1257RC	+	+	++	+/−
MZ2514RC	+	+	++	+/−
MZ2733RC	+	+	++	+/−
MZ2877RC	+	+	++	+/−

+/− : 5-20% positive; +: 20-50% positive; ++ : > 50-100% positive; +++: 100% positive, and staining intensity > 10 fold to isoytpe control.

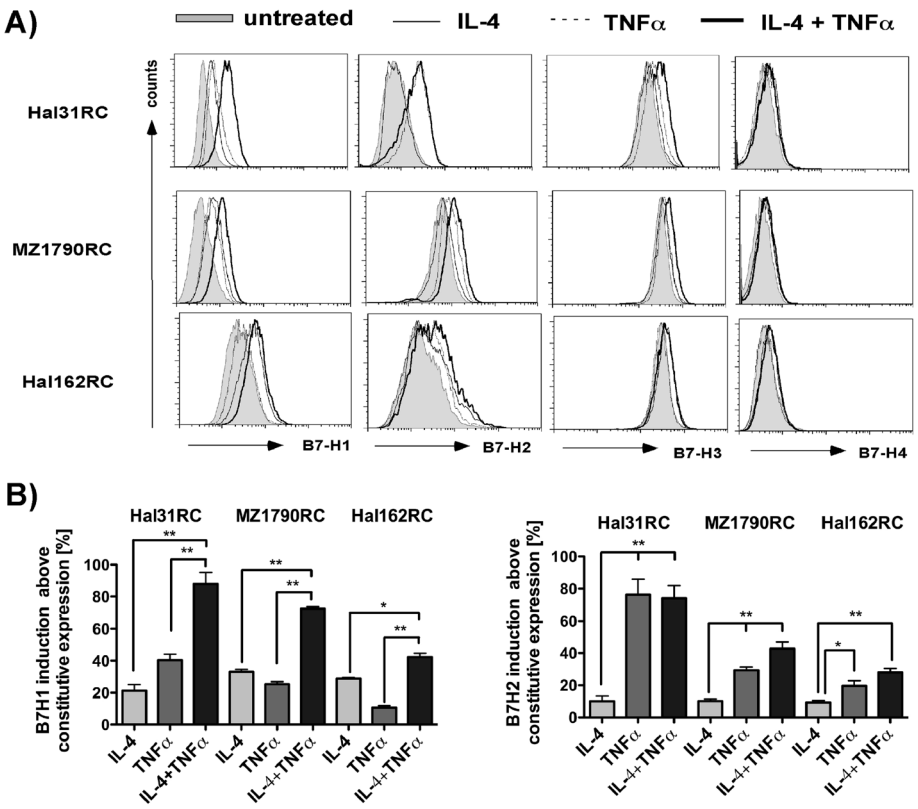

Figure 2 Significant increase of B7-H1 surface expression by IL-4 and TNFα treatment of RCC cell lines. Different RCC cell lines (Hal31RC, Hal161RC, MZ1790RC) were treated with either IL-4 or TNFα alone before B7-H expression was determined by flow cytometry as described in Materials and Methods at 72 hrs. **A)** Shown are FACS histogram overlay plots for B7-H1, B7-H2, B7-H3 and B7-H4. Each plot shows overlay graphs, depicting constitutive protein expression and expression upon single or combined cytokine treatment. Representative data of at least 3 different experiments are shown. **B)** Cytokine-mediated induction of B7-H1 and B7-H2 in % using constitutive expression levels as reference value. Combined data of 3 different experiments are given.

on B7-H1 protein expression (Figure 2 and Figure 3A), with a < 6 fold increase in RCC31 cells (Figure 3A). Furthermore, IFNγ treatment of RCC cells increased B7-H1 mRNA and protein expression (data not shown) confirming recent reports [19].

In addition, other cytokines like IFNα, IFNβ, TGFβ, IL-6 and IL-10 were tested for their ability to modulate B7-H expression on RCC cells, but none of them altered the expression levels of these molecules. The lack of the cytokine-mediated effects on B7-H family members was specific, since these cytokines modulate e.g. HLA class I molecule expression (data not shown).

Transcriptional control of cytokine-mediated induction of B7-H1

To identify the kinetics of IL-4- and TNFα-mediated regulation of B7-H1 expression RCC cells were treated for different time points with IL-4 and TNFα alone or in combination before qPCR and flow cytometry was performed. Already 4 hrs post combined cytokine treatment a significant induction of B7-H1 mRNA was detected (Figure 3B). Treatment with IL-4 alone only marginally increased the B7-H1 transcript levels throughout the time points tested, whereas TNFα alone induced a significant increase in B7-H1 mRNA levels at 24 hrs post treatment. Combined cytokine treatment had always the strongest impact on B7-H1 mRNA (Figure 3B). An enhanced protein expression was only detected at later time points, with a maximal induction at 72 hrs post cytokine treatment. These results pointed towards a regulation at the transcriptional rather than posttranscriptional level, e.g., by protein stabilization.

In order to test B7-H1 regulation at the transcriptional level in more detail, promoter binding prediction program (TESS) was used to determine direct binding sites for STAT6 (downstream of IL-4 signaling) and NFκB (p65, downstream of TNFα) on the B7-H1 promoter (−952 to −1 bp before transcript start). A weak binding site for STAT6 (TTACAAGAA) and two overlapping high affinity binding sites for NFκB (GGAAAGTCCA; AGGAAAGTCCAAC) were found in the B7-H1 promoter. One NFκB binding site in the B7-H1 promoter

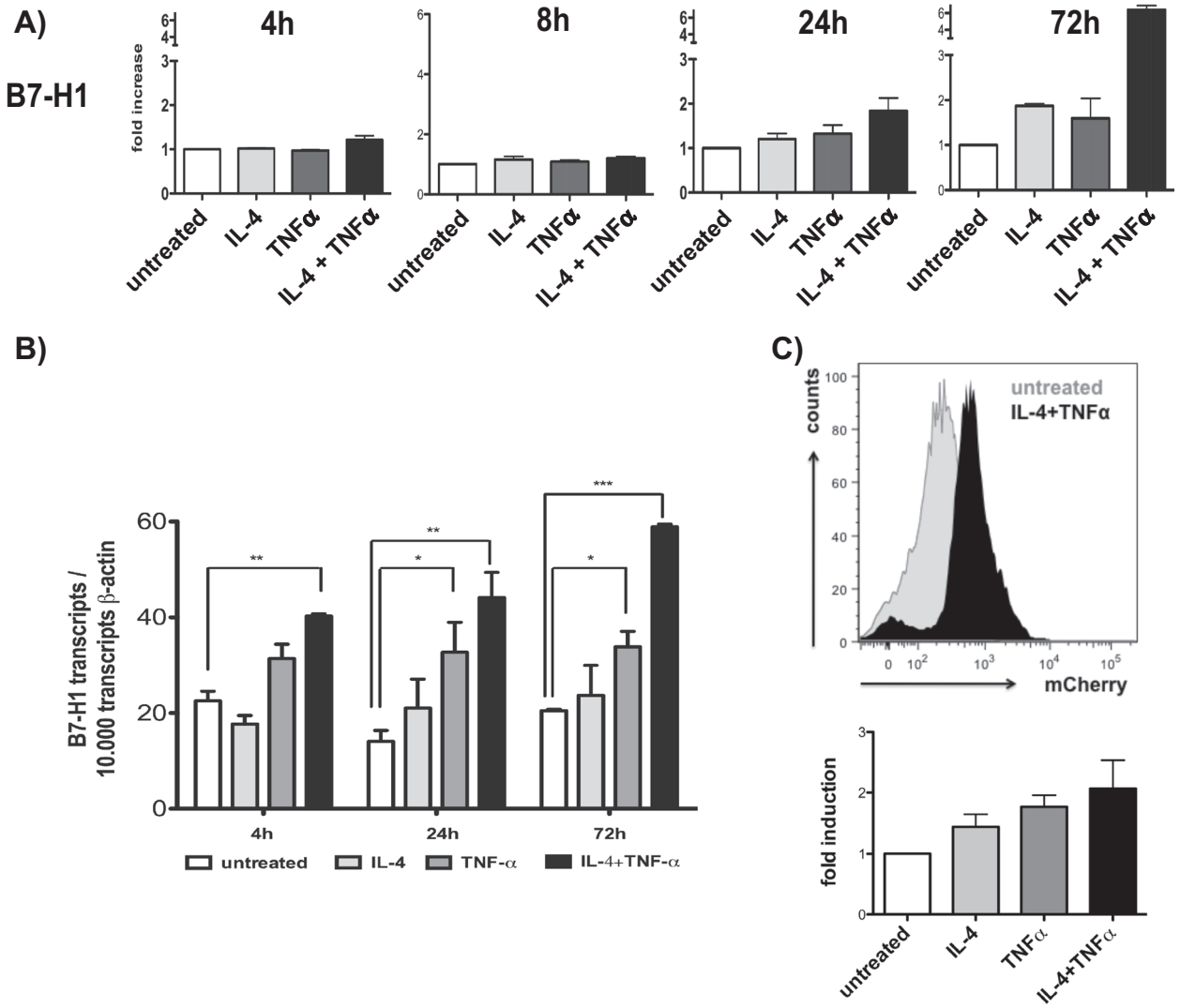

Figure 3 Time kinetic of B7-H1 expression and B7-H1 promoter targeting upon combined cytokine treatment with IL-4 and TNFα. A) Hal31RC was treated with either IL-4 or TNFα alone and with the combination of the two cytokines. B7-H1 protein expression was analyzed at the time points indicated by flow cytometry. Shown are fold increase in B7-H1 expression compared to untreated cells. Representative data of at least 3 different experiments are shown. **B)** B7-H1 mRNA expression in Hal31RC upon treatment with either IL-4 or TNFα alone and with the combination of the two cytokines for the time points indicated are shown. Using a B7-H1 plasmid as standard, B7-H1 transcript levels per 10.000 transcripts β-actin are shown. Data are obtained by real time PCR. Representative data of 3 different experiments are shown. **C)** BUF1088Mel was transfected with the mCherry B7-H1 promoter construct and stimulated with IL-4 and TNFα. The mCherry fluorescence was measured after 72 hrs by FACS and a representative histogram plot is depicted. The bar graph shows the combined result of a total of 3 independent assays.

has been shown to control B7-H1 expression in renal tubular cells [24].

Based on this finding B7-H1 promoter studies were performed [25]. The wild type B7-H1 promoter construct hooked to the mCherry fluorescent protein was stably transfected into BUF088Mel cells (Additional file 1: Figure S1). Transfectants were subsequently left untreated or treated with either cytokine (IL-4 or TNFα) alone or in combination before promoter activity was determined 72 hrs later. As shown in Figure 3C, an enhanced B7-H1 promoter activity as determined by flow cytometry of the mCherry fluorescence was found upon single and more pronounced upon combined treatment with IL-4 and TNFα. This suggests a transcriptional upregulation of B7-H1, which might be mediated by activation of STAT6 and NFκB.

Alterations in pSTAT6, NFκB, LFA-1, CD40 and HLA class I antigen expression levels upon treatment of RCC cells with IL-4 and TNFα

In addition to the regulation of B7-H molecules by cytokine treatment downstream signal cascade components and other processes known to be of relevance for the T

cell/tumor interaction and the growth behavior of RCC cells were investigated.

A growth inhibition of RCC cells was detected upon IL-4 addition, which was not further pronounced in the presence of TNFα (Additional file 3: Figure S3). In contrast, TNFα alone had no influence on the cell proliferation, which is in discrepancy to earlier published data showing an increased proliferation upon TNFα treatment, but a strong influence of the culture media conditions were found [26].

Following IL-4 treatment alone or in combination with TNFα phosphorylation of STAT6, a key signal molecule downstream the IL-4 receptor, was detected by flow cytometry (Figure 4A). A known key downstream effector of TNFα signaling is represented by NFκB. An enhanced expression and activation of NFκB was found in TNFα as well as TNFα- and IL-4-treated RCC cells (Figure 4A). Next to the upregulation of NFκB the inhibitory subunit of NFκB, named IkB, was downregulated upon TNFα treatment in RCC cells (data not shown). Downregulation/degradation of IκB as a part of the classical NFκB pathway activation has been described earlier [27].

Additional important molecules for T cell/tumor communication are CD40, a costimulator of the TNF-R family, the adhesion molecule CD54 (LFA-1) and HLA class I antigens. As shown in Figure 4B all these surface molecules were upregulated by the treatment of RCC cells with IL-4 and TNFα, either alone or in combination. The combined treatment of IL-4 and TNFα had an additive (CD40, HLA class I) or a synergistic effect (LFA-1) on the protein expression, respectively (Figure 4B).

B7-H1 blocks allospecific T cell proliferation

B7-H1 has been shown to modulate T cell responses. [19,28] To test the functional consequences of increased B7-H1 on RCC cells upon IL-4 and TNFα treatment co-culture assays of tumor cells with allospecific T cells were performed. A decreased $CD8^+$ T cell proliferation was detected upon co-cultivation of T cells with IL-4 pretreated as well as with IL-4 and TNFα pretreated tumor cells (Figure 5A and B). The direct influence of both cytokines on T cells was eliminated by 3 times washing of cytokine-treated tumor cells, prior to co-culture assays. The decreased proliferation of $CD8^+$ T

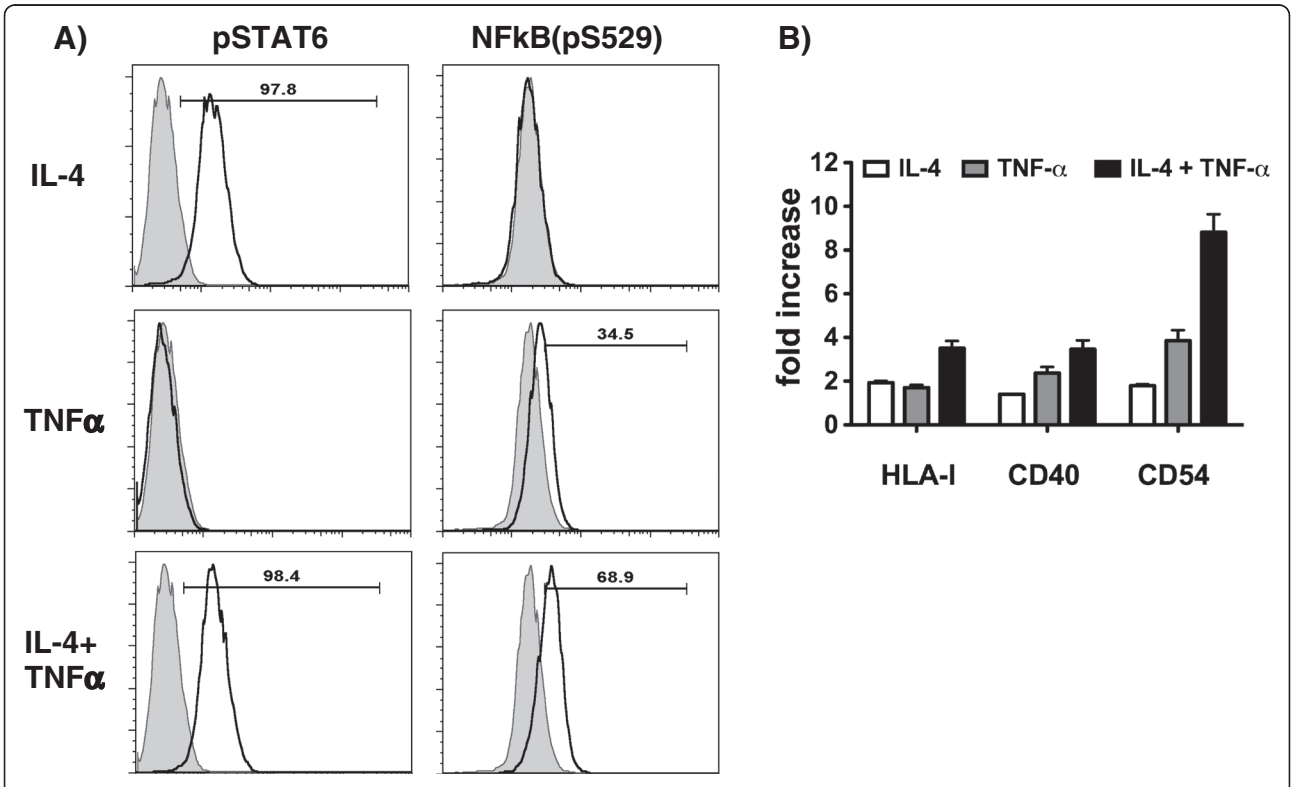

Figure 4 Involvement of signaling components and change of HLA-I, CD40 and CD54 expression upon combined cytokine treatment with IL-4 and TNFα in RCC cell lines. Hal31RC was treated with either IL-4 or TNFα alone and with the combination of the two cytokines. **A)** Intracellular pSTAT6 as well as NFκB (pS529) at 30 min post stimulation were analyzed by flow cytometry. Gray filled graph represents constitutive expression and black bold line represents cytokine-induced expression of either signaling component. Numbers on gates are percentage of positive cells compared to medium control. Representative data of at least 5 different experiments are shown. **B)** HLA class I, CD40, CD54 expression 72 hrs post cytokine treatment were analyzed by flow cytometry. Data are presented as fold increase when compared to constitutive expression of these molecules on untreated Hal31RC. Data are mean ± SEM of 9 different experiments.

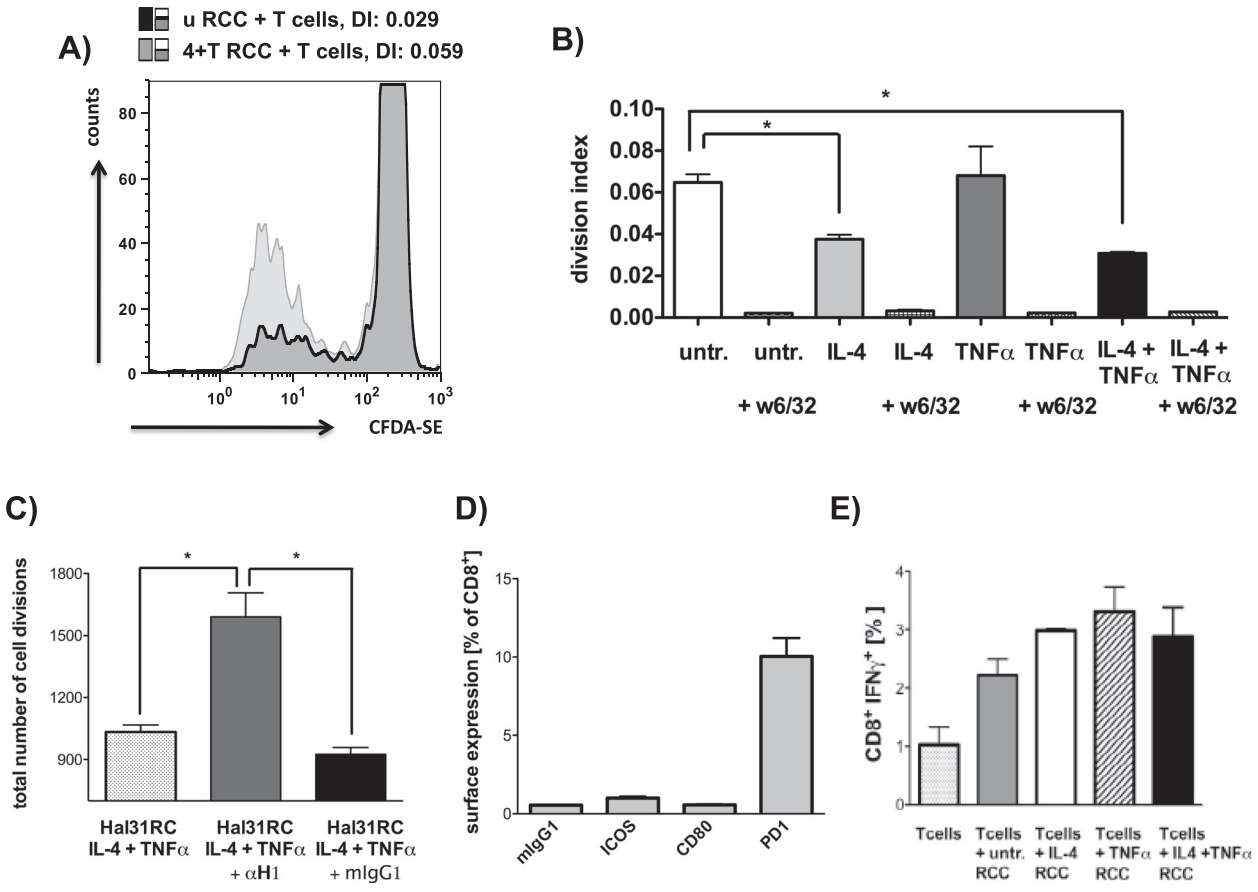

Figure 5 Block of allogeneic CD8$^+$ T cell proliferation upon coculture with RCC cells pretreated with IL-4 and TNFα. A-C) Hal31RC cells were treated with either IL-4 or TNFα alone and with the combination of both cytokines. CD8$^+$ T cells from allogeneic PBMCs were sorted, CFDA-SE labeled and cocultured with cytokine pretreated RCC31Hal. **A)** Representative FACS histogramm plots of proliferated CD8$^+$ Tcells cocultured with untreated (u RCC) as compared to IL-4 or TNFα pretreated Hal31RC (4 + T RCC) are shown. The division index (DI) is given. **B)** Summary of CD8$^+$ T cell proliferation on cytokine pretreated Hal31RC. The division index (DI) is given. Pan anti-HLA class I antibody (w6/32) served as specificity control. Data are means of 4 different donors ± SEM. **C)** Partial reversal of T cell proliferation inhibition upon coculture of CD8$^+$ T cells with anti-B7-H1 antibody or respective isotype control preincubated Hal31RC. Hal31RC were pretreated with IL-4 and TNFα for 3 days. Data represent the mean of 3 different donors ± SEM. **D)** Analyses of constitutive B7H ligand expression, ICOS, CD80 and PD-1 on allogeneic CD8$^+$ T cells prior to coculture with RCC31. Data represent means of 7 different donors ± SEM. **E)** IFNγ production of isolated CD8$^+$ T cells upon coculture of cytokine pretreated Hal31RC cells. Data are means of 3 different donors ± SEM.

cells upon co-culture with IL-4- and TNFα-pretreated tumor cells could be partially converted by the use of an anti-B7-H1 blocking antibody as proof for the inhibitory role of B7-H1 on tumor cells (Figure 5C).

To identify the possible binding partner of B7-H1 on T cells, an anti-PD1 antibody was used for staining to detect the most likely candidate PD1. Indeed, PD1 was expressed in 10.05 ± 1.17% of CD8$^+$ T cells analyzed (Figure 5D). Since CD80 expression on T cells has been postulated [29] and CD80 can additionally bind B7-H1 [30] its CD80 expression was determined on T cells. In addition, since B7-H2 expression is known to be increased upon treatment with TNFα, ICOS, the ligand of B7-H2 was analyzed on CD8$^+$ T cells prior to co-culture assays. As shown in Figure 5D, CD80 and ICOS were only very weakly expressed on CD8$^+$ T cells.

Important CD8$^+$ T cell effector functions are cytokine production and the ability to kill target cells. As determined flow cytometry IFNγ secretion by CD8$^+$ T cells was unaltered upon co-culture with cytokine-treated RCC cells (Figure 5E). Furthermore, the killing ability of CD8$^+$ T cells was determined by measurement of CD107a degranulation, CD8$^+$ T cells upregulated CD107a expression upon co-culture with RCC cells, but no specific changes with respect to co-culture with cytokine pretreated tumor cells were found (data not shown).

Discussion

The present study was undertaken to discover the distribution and regulation of B7-H family members in RCC by cytokines released from immune cells of the tumor microenvironment. Interesting, a synergistic increase of

B7-H1 surface expression in RCC cells upon treatment with IL-4 and TNFα was found. As early as 4 hrs after treatment, B7-H1 mRNA was significantly enhanced resulting in 6- fold increase in protein surface expression by 72 hrs and was mediated by an upregulation of the B7-H1 promoter activity by combined IL-4 and TNFα treatment. In addition, the increase in B7-H1 protein surface expression on RCC cells was associated with a decreased allospecific T cell proliferation upon co-culture experiments.

Detailed analysis showed a constitutive, but variable surface expression of B7-H1 to B7-H4 molecules on RCC cells. Expression of B7-H1, B7-H3 and B7-H4 *in situ* has been correlated with a worse clinical outcome of RCC patients [9,31,32]. In all tested RCC cell lines, B7-H4 was rather weakly expressed. In contrast B7-H3 was strongly expressed on RCC cell lines. To the best of our knowledge, we are the first to show B7-H2 expression on RCC cells. There exist random reports showing expression of this molecule in human tumors such as glioblastoma [33] and melanoma [34]. B7-H2 on glioma cells leads to an increase in T cell-mediated anti-tumor immunity [35].

In addition, B7-H1 expression on RCC cells was confirmed with a weak or intermediate expression level by all RCC cell lines tested. Testing of the constitutive cytokine expression revealed a weak TNFα production by one RCC cell line, while all others were negative. These data are in line with earlier reports describing that some RCC cells are able to produce TNFα [36]. Furthermore, RCC cells in our study lack IL-4 expression thereby confirming previous published data [37]. A prerequisite to respond to a particular cytokines is the expression of the respective cytokine receptors. Both the IL-4R as well as TNFRI expression was found on all RCC cells tested. TNFα acts via TNFRI and II both expressed on RCC cells [38]. IL-4R expression on RCC cells *in vitro* and *in situ* has been demonstrated before [39]. Interestingly, structural differences for IL-4R on RCC cells when compared to immune cells exist, which might partially explain the differential outcome of IL-4 action in these cells [37]. Most of the RCC cell lines tested in the present study showed a very good and reliable response to IL-4 and TNFα treatment as demonstrated by phosphorylation of STAT6, enhancement of pNFκB and downregulation of IkB.

Given the importance of B7-H molecules for the outcome of RCC patients and the presence of cytokines in the tumor microenvironment, the regulation of these family members upon treatment with various cytokines was determined. As already described, upregulation of B7-H1 expression by IFNγ was confirmed [19]. In addition the most prominent effect on the regulation of B7-H1 was found using combined IL-4 and TNFα treatment. B7-H1 was transcriptionally controlled by these cytokines. B7-H2 was only upregulated by TNFα treatment, but not by IL-4.

IL-4 and TNFα can both be produced by different immune cells and thus represent components of the tumor microenvironment. In RCC TNFα is produced by tumor-associated macrophages (TAM) [40]. TAMs can be subdivided into classical M1 phenotype macrophages that produce and dependent on proinflammatory cytokines, such as TNFα and into alternative M2 phenotype macrophages [41]. M2 macrophages are characterized by the production of IL-10, TGF-β and are induced by IL-4. The ratio of M1/M2 TAMs together with the number and phenotype of dendritic cells, myeloid derived suppressor cells (MDSC) and the Th1/Th2 balance determine the cytokine milieu and thereby the anti-tumor response in the tumor microenvironment. Already 15 years ago $CD4^+$ Th cells of the Th1 (predominantly IFNγ) and Th2 (predominantly IL-4) cells as well as $CD8^+$ T cells have been shown to play a key role for an effective anti-tumor response. Furthermore, IL-4 has a tremendous impact on the anti-tumor immunity by shifting the Th1/Th2 balance [42]. The importance for IL-4 in RCC is demonstrated by the existence of a functional polymorphism in the IL-4 gene (−590 T) leading to an enhanced expression of this cytokine, which is correlated with an increased risk of developing RCC [43] and a decreased survival [44] when compared to RCC patients carrying the other haplotype (−590C). In contrast to earlier reports, IL-4 can reduce tumor growth suggesting that the time point and local distribution of high IL-4 levels have an impact on RCC progression. Of importance, microarray data reveal a positive correlation of B7-H1 with TNFα, NFkB and STAT6 (http://r2.amc.nl) in kidney tumor tissue in vivo, nicely supporting our data of a linked B7-H1 expression with these cytokines.

In addition to IFN-γ, the regulation of B7-H expression by cytokines has been studied earlier, but mostly on immune cells and not on RCC cells. Kryzek and co-authors showed an increase of B7-H4 in TAMs upon treatment with IL-6 or IL-10 [45]. Similar to our findings TNFα upregulates B7-H2 on embryonic fibroblasts [46] and endothelial cells [47], while IL-4 did not modulate B7-H2 and the combined treatment had no additional effect [47]. Interestingly, on human endothelial cells TNFα together with IFNγ synergistically affect induction, whereas TNFα alone did not induce any B7-H1 expression [48].

As already proposed by and confirmed in this study combined IL-4 and TNFα treatment exerts a synergistic effect on the increase of HLA class I antigen expression in RCC cells, which might enhance T cell-based anti-tumor responses [13]. This was further supported by an increased expression of components of the APM leading to increased HLA class I surface antigen expression in RCC cells upon IFN-γ treatment [30]. Since another

hallmark of effective T cell response is cell adhesion, ICAM1 expression was analyzed. ICAM1 expression was highly upregulated upon combined IL-4 and TNFα treatment, which can support T cell/tumor interaction. This is in line with published data demonstrating that TNFα induces ICAM1 expression [49].

As functional consequences of cytokine-mediated enhanced B7-H1 on RCC tumor cells, a decreased allospecific $CD8^+$ T cell proliferation was found, which could be partially converted by addition of an anti-B7-H1 antibody. Since PD1 was the only substantially expressed receptor on the $CD8^+$ T cells used in the co-culture assays, it is therefore most likely responsible for this effect. This assay nicely resembles the *in vivo* situation, since enhanced PD1 expression on tumor-infiltrating immune cells has been found in RCC and could be associated with poor patients' prognosis [50]. However, IFN-γ and CD107a mobilization was not altered in $CD8^+$ T cells upon co-culture with cytokine pre-treated tumor cells. An inhibitory effect of B7-H1 on $CD8^+$ T cells has already been described in different studies. A block in T cell proliferation owing to B7-H1 has been shown before with overexpression or blocking of B7-H1, but not with cytokine-pretreated cells [51,52,20]. Additionally, a direct decrease of $CD8^+$ T cell killing and cytokine production upon co-culture assays with anti-B7-H1 blocking antibody for human TCR tg $CD8^+$ T cells in RCC has been found [19]. On the other hand, Dong and coworkers (2002) showed that TCR tg human CTL are equally able to kill B7-H1 over-expressing melanoma cells, but a B7-H1 dependent induction of T cell apoptosis was detected [28]. Together with the findings of this study the data demonstrate the powerful influence of B7-H1 on the modulation of different T cell effector responses, which highly appear to depend on the co-culture systems chosen.

The observed block of T cell proliferation on $CD8^+$ T cells co-cultured with single IL-4-treated RCC cells could be due to an additionally interaction of PD1 on T cells with its second ligand B7-DC. A weak constitutive expression of B7-DC was found that could be also a subject to regulation by IL-4 similar as shown for macrophages [53] possibly not to TNFα as analyzed for monocytes [52]. B7-DC has been shown to inhibit human T cell proliferation by PD1 binding [51].

High dose TNFα has been used to treat solid tumors, but due to many side effects upon systemic administration the success rate has been rather low, and strategies to administer this cytokine more locally had been developed for e.g. melanoma patients [54], but have not been used to treat RCC.

Phase II clinical trials using IL-4 for the treatment of RCC were not beneficial for these patients [16,18]. This might be at least partially explained by the decreased T cell proliferation capacity upon co-culture assays with IL-4-treated RCC cells. TNFα alone had no effect on T cell effector responses in the setting used in our study, although TNFα treatment exerted beneficial anti-tumor effects in a xenograft mouse model with RCC tumors [12].

Conclusion

Concluding the data, this study showed for the first time a detailed analysis of B7-H molecule regulation upon cytokine treatment in RCC. B7-H1 exhibited the strongest sensitivity to IL-4 and TNFα by a synergistic upregulation in RCC cells and is controlled at the transcriptional level by direct promoter targeting. The importance of this B7-H1 induction is demonstrated by an inhibition of T cell proliferation thereby contributing to the proposed significance of B7-H1 in cancer immunity. The study furthermore supports the rational of using B7-H1/PD1 checkpoint antibodies for the treatment of tumor patients.

Additional files

Additional file 1: Figure S1. Reporter gene plasmid map for B7-H1.

Additional file 2: Figure S2. B7-H4 mRNA as determined by real time PCR is given. Transcript numbers are calculated using a β-actin and B7-H4 plasmid as template. Representative data from 2 experiments are shown.

Additional file 3: Figure S3. Growth inhibition of cells treated with IL-4 and the combination of IL-4+TNFα for 3 different RCC cell lines is shown. Same numbers of cells were seeded into wells and cells were equally detached and counted using trypan blue exclusion at the end of culture time (72hrs). Combined data of three different experiments are shown.

Abbreviations

APM: Antigen processing machinery; CTL: Cytotoxic T lymphocyte; NFκB: Nuclear factor kappa B; IFN: Interferon; IL: Interleukin; luc: Luciferase; mAb: Monoclonal antibodies; MHC: Major histocompatible complex; RCC: Renal cell carcinoma; STAT: Signal transducer and activator of transcription; TAA: Tumor-associated antigens; TAM: Tumor-associated macrophages; TCR: T cell receptor; TNF: Tumor necrosis factor.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

DQ carried out main part of experiments, interpretation of results and wrote the manuscript, SJ-B carried out the promoter studies, UM performed part of the FACS analysis and the real time PCR experiments, BSc performed part of the T cell assays and BSe conceived of the study, participated in its design and coordination and was involved in writing the manuscript. All authors read and approved the final manuscript.

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Human Cancer Biology

Clinical Cancer Research

B7-H4 Expression in Human Melanoma: Its Association with Patients' Survival and Antitumor Immune Response

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Abstract

Purpose: Cancers have developed a number of strategies to escape immune responses including the differential expression of costimulatory molecules of the B7 family. B7-H3 and B7-H4 have recently been described in different tumor entities but the relevance for melanoma has not yet been studied so far.

Experimental Design: Using immunohistochemistry, B7-H3 and B7-H4 expression was studied on 29 melanoma lesions. Survival curves and log-rank tests were used to test the association of protein expression with survival. Cell lines were evaluated for B7-H3 and B7-H4 expression by PCR and flow cytometry. Functional T-cell–tumor coculture assays were carried out with *in vitro* generated tumor transfectants.

Results: B7-H3 and B7-H4 expression was detected in primary tumor lesions (29 of 29 and 28 of 29) and in metastases (28 of 29 and 26 of 29). The numbers of $CD68^+$ macrophages were significantly lower in patients with low B7-H4 expression, whereas $CD8^+$ T-cell infiltrates were independent of expression levels. Furthermore, a survival benefit for patients with B7-H4 low expressing melanoma was found, whereas B7-H3 was not associated with any clinical parameter. All 23 melanoma cell lines analyzed expressed B7-H3 and B7-H4 mRNA and protein, but B7-H4 was restricted to intracellular compartments. On silencing of B7-H3 by specific shRNA tumor-associated antigen–specific T cell responses were unaltered. Overexpression of B7-H4 on melanoma cells did not alter the cytotoxicity of different $CD8^+$ effector cells, but drastically inhibited cytokine production.

Conclusions: Our study provides for the first time evidence of B7-H4 expression on melanoma cells as a mechanism controlling tumor immunity which is associated with patients' survival. *Clin Cancer Res; 17(10); 3100–11. ©2011 AACR.*

Introduction

Malignant metastatic melanoma represents a severe tumor disease with a bad prognosis. Current treatment regimens are often not very successful, in particular, in patients with unresectable stage III and IV melanoma. Therefore, ongoing research aims in getting new insights into this disease thereby concentrating on the interplay with the immune system. For a number of reasons, melanomas are considered as immunogenic tumors due to (i) the existence of immune-cell infiltrates that are associated with better patients' outcome (1–7), (ii) the relatively high incidence of spontaneous tumor regression, (iii) the description of numerous tumor-associated antigens (TAA) and respective T-cell responses over the years (4, 5), and furthermore, (iv) the responsiveness of melanoma patients to immune therapies, such as cytokines like interleukin 2 (IL-2; ref. 6) and IFN-α (7), αCTLA-4 treatment (8) or combined strategies of adenovirus encoding MART-1 along with IL-2 applications (9).

The activation of T cells is controlled by 2 signals. The first is provided by the binding of the specific T-cell receptor (TCR) on T cells to a peptide–HLA complex on antigen-presenting cells (APC) or HLA class I–positive tumor cells. The second signal for proper T-cell activation is given by costimulatory molecules (10). The outcome of costimulation can either be costimulatory or coinhibitory, which highly depends on the time of expression of costimulators, receptor availability, and on the status of the T cells. Whereas the prototype of costimulatory molecules of the B7 family, B7-1 and B7-2, are predominantly influencing naive T-cell functions (11), the more recently identified members of this family, the B7-H molecules, predominantly control the effector phase of T-cell responses (12). The growing B7-H family comprises of many surface molecules, among them are B7-H1 (PD-L1), B7-H2 (ICOS-L), and B7-H3 and B7-H4 (B7x, B7S1; ref. 11). Even though many studies have shown an inhibitory role for B7-H4 (13) in the setting of progressive renal disease, a costimulatory activity of B7-H4 has also been proposed (14). So far, the

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Translational Relevance

Some members of the B7 family are expressed in tumors of distinct origins and appear to be involved in escape from immunosurveillance. We describe for the first time B7-H3 and B7-H4 expression in primary and metastatic melanoma lesions. Tumor B7-H4 expression was associated with a poor survival of melanoma patients. To understand the functional role of tumor B7-H4, we carried out HLA-I–restricted T-cell–tumor coculture assays. On B7-H4 overexpression, TAA-specific T-cell–cytokine response was inhibited, showing the importance of B7-H4 for the anti-melanoma immune response. The knowledge of differential B7-H4 expression and the subsequent effect for the antitumor immune response could be of great help for the treatment of melanoma patients.

receptor for B7-H4 has not been identified (12). B7-H3 has been shown to promote T-cell responses possibly via an activating receptor called TLT-2 (15) and on the contrary to inhibit T-cell responses by a not yet identified inhibitory receptor (16).

In the last decade, members of the B7-H family have been shown to be expressed in different tumor entities, mostly with an adverse clinical association (17). In melanoma, high levels of B7-H1 expression have recently been shown to be positively associated with reduced patient's survival (18) extending earlier studies on the role of B7-H1 for the antitumor immune response (19), whereas for B7-H2 (ICOS-L) *in situ* expression data are available, but the clinical significance is unclear (20). Tumor-associated B7-H3 has been described in a number of different tumor entities, for example, in prostate and renal cell cancer (RCC) and neuroblastoma (12). The level of expression of B7-H3 correlates to different clinical outcomes; whereas in gastric cancer patients (21), high expression levels of B7-H3 were associated with increased patients' survival, the level of B7-H3 negatively interferes with patients' outcome in RCC (22). The role of B7-H3 in tumor immunity is therefore under debate and in particular, its function in human antitumor $CD8^+$ T-cell responses has not been analyzed in detail. Similarly, B7-H4 expression has also been shown in a number of different tumor entities, such as RCC, ovarian, and breast cancer (17). B7-H4 is mostly associated with poor patients' outcome. The role for B7-H4 in the direct interaction of tumor cells with $CD8^+$ T cells has hardly been studied so far. Because melanoma lesions are often infiltrated by immune cells with diminished effector functions (23), the antimelanoma immune response could also be controlled by the immunosuppressive action of costimulatory molecules expressed on tumor cells.

To elucidate the role of B7-H3 and B7-H4 in melanoma, patients' material as well as cell lines were assayed for *in vivo* and *in vitro* expression data and clinical significance. B7-H3 and B7-H4 were found to be expressed in primary lesions and in metastases of stage III and IV metastatic melanoma patients. No clinical relevance of B7-H3 expression was detected, but patients with B7-H4 low expressing primary melanoma had a survival benefit. In line with the patients' data, *in vitro* studies showed that tumor-associated B7-H4 inhibits cytokine production of different antitumor effector T cells in T-cell–tumor coculture assays. These data show for the first time B7-H3 and B7-H4 expression in melanoma lesions and more remarkably show a functional consequence of B7-H4 for the patients' outcome and an *in vitro* anti tumor response in metastatic melanoma.

Materials and Methods

Patients' material

From the histology database of the Department of Dermatology and Venereology of the University of Halle-Wittenberg (Halle, Germany), patients' samples were retrospectively selected, from whom a histologically preserved primary tumor (cutaneous malignant melanoma), samples from cutaneous or subcutaneous (lymph node) metastases as well as material of a benign cutaneous lesion were available. Twenty-nine of the cases fulfilled this criteria and 26 of the patients belong clinically to stage III and 3 of the patients to stage IV melanoma. Among the patients, 16 (B7-H3) or 17 (B7-H4) were men and 13 or 12 women, respectively, with a mean age of 60.9 (27–84) at the primary tumor resection. The 29 cases could be classified into 17 superficial spreading melanoma (SSM), 5 nodular melanoma (NM), 2 acral lentiginous melanoma (ALM), and 5 unclassified melanoma (UCM) with a mean Breslow index of 4.23 (0.65–9). The mean appearance of melanoma metastases was 1.88 (0–7.4) years after primary tumor resection and metastases used for histological sections were grouped into 3 cutaneous, 4 subcutaneous, 12 lymph node, and 10 nonspecified (close to cutaneous) metastases. The benign lesions comprise melanocyte-derived naevi, epidermal cyst, and acanthomes. The Ethics Committee at the University Hospital in Halle (Germany) approved this study.

Immunohistochemistry

Five-microgram thick paraffin-embedded serial sections were stained for melanoma markers, B7-H3 and B7-H4 and immune cell infiltrates using a Ventana Benchmark-XT system. Counterstaining of sections with hematoxylin (Ventana Medical Systems Inc.) was carried out. The following monoclonal antibodies (mAb) were used: αHMB-45 (Dako), αMelan-A A103 (Dako), αCD4 (Neomarkers), αCD8 (Dako), αCD56 (Novocastra Laboratories Ltd.), αCD68 (Dako), α-podoplanin (clone D2-40, Dako), αB7-H3 (5 μg/mL) from R&D Systems and αB7-H4 (10 μg/mL), clone H74, from eBioscience. Stainings were carried out essentially as described (24), with the exception that the ultraView Universal Alkaline Phosphatase Red Detection Kit (Ventana Medical Systems Inc.) was used as detection system. The immune cell infiltrates were evaluated in a semiquantitative fashion using a scoring from

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0 to 3 (for CD4, CD8, and CD56), 0: no positive cells detected; 1: less than 5% positive cells of total infiltrate; 2: multifocal diverse single cells and groups; 3: like in 2 plus tumor underlying lymph infiltrate. CD68-positive cells are given as percent positive cells of the immune infiltrate in the tumor section. Dermatopathologists evaluated the expression of B7-H3 and B7-H4 in histological sections in a blind fashion. Results were scored as 0 (negative), 1 (heterogeneous), and 2 (positive) when the percentage of stained tumor cells was less than 25, between 25 and 75, and more than 75, respectively. In addition, the staining intensity was scored as 0 (negative), 1 (weak), 2 (moderate), and 3 (strong). The results were then analyzed according to Armes and colleagues (25) combining the scores for frequency and quality. Combined scores of 0 to 5 were given distinguishing low (scores 0–3) and high (scores 4 and 5) B7-H3 and B7-H4 expression, respectively. B7-H3 and B7-H4 stainings were initially established on tonsilar tissue.

The specificity of the stainings in the tumor lesions was controlled by using the mIgG1 isotype control antibody (AbD Serotec) and by employing a competition assay. The competition assay was carried out by preincubation of 75 μg/mL of recombinant mouse B7-H4 protein (R&D Systems) with the B7-H4 antibody for 45 minutes at room temperature before following the staining protocol. In addition, the D2-40 antibody, which stains lymph vessels, but neither tumor cells nor immune cells in the sections was used.

Cells, cell lines, reagents, antibodies, and peptides

The 23 melanoma cell lines analyzed have been recently described (26) and were either a kind gift from S. Ferrone or from the European tumor cell line data base (ESTAB project; see www.ebi.ac.uk/ipd/estdab). The RCC cell line Hal31RCC and MZ2733RCC were generated in house from tumor lesions of clear cell RCC patients. Epstein-Barr virus (EBV)-transformed HLA-A02 restricted B cells were generated by Wölfel and colleagues (27) and Bukur (in house) from healthy blood donors. SKOV3, SKBR3, and T2 cells were obtained from the American Tissue Culture Collection (ATCC). Tumor cell lines were cultured as described before (26, 28). Peripheral blood mononuclear cells (PBMC; HLA-A02$^+$) from healthy donors were obtained from the blood bank of the university hospital.

IL-2 (Proleukine, Pharmacy, University of Halle), Triton X-100 (Merck KGaA), phorbol myristate acetate (PMA), propidium iodide, and ionomycin from Sigma-Aldrich were used. SureSilencing shRNA (short hairpin RNA) plasmids for human B7-H3, were purchased from SABioscience. αCD8 microbeads was obtained from Miltenyi Biotech.

The following antibodies for cell culture were employed: αCD3 (clone OKT3) from eBioscience), purified mIgG2a from (Millipore), and αHLA-I (clone w6/32) obtained from culture supernatants of hybridomas (28).

Monoclonal antibodies (mAb) for flow cytometric analysis were: αCD8, αHLA-I (clone B9.12.1), αCD25, αCD45RO, αICAM-1 from Beckman Coulter; αCD28 and αCD27 from Becton Dickinson; αB7-H4 from AbD serotec; αB7-H4 as well as was αCD45RA from eBioscience; αCCR7 as well as αB7-H3 were used from R&D systems. Respective isotypes were purchased from BD Bioscience or Beckman Coulter. The antibodies used were unconjugated and/or as direct conjugates with FITC, Alexa-488, PE, APC or PE-Cy7. HLA-A2–restricted peptides, $Tyr_{368–376}$ pep (seq. YMNGTMSQV; ref. 29), and $MART_{27–35}$ pep (seq. AAGIGILTV; ref. 30) were synthesized by Peptides and Elephants.

Flow cytometry

Flow cytometric life cell analyses were essentially carried out as described before (26). The specificities of the B7-H3 and B7-H4 stainings were controlled by preincubation of cells with a 100× fold excess of the respective unconjugated antibody or irrelevant mouse IgG1. Forward/side scatter gating and propidium iodide staining in life cell analysis excluded dead cells. Intracellularly protein expression was detected as described before (31). Cytometric analyses were carried out using a FACSscan (Becton Dickinson) or FC500 (Beckman Coulter) flow cytometer and CellQuest or CXP and FlowJo (Tree Star) software.

RT-PCR

Total cellular RNA from frozen cell pellets was extracted using RNAeasy Mini Kit (Quiagen) and reversely transcribed into cDNA (Fermentas). Semiquantitative reverse transcriptase PCR (RT-PCR) from cellular RNA was carried out using the following oligonucleotide primers: for B7-H4 forward, 3′-aggcttctctgtgtgtctcttc-5′ and reverse, 3′-cttgctcttgtttgctcactcc; for B7-H3 forward, 3′-ctgtctgtctcattgcactgc-5′ and reverse: 3′-caggctatttcttgtccatcat-5′; and for β-actin forward, 3′-tcctgtggcatccacgaaact-5′ and reverse: 3′-gaagcatttgcggtggacgat-5′.

Transfection and generation of stable transfected cell lines

B7-H4 was cloned after RT-PCR from mRNA of MZ2733RCC cells into pCMVIrNeo vector (32) following transfection of melanoma cells. In brief 3×10^5 BUF 1088 cells were seeded in 6-well plates and transfected the following day using Lipofectamine 2000 (Invitrogen) with 3 μg plasmid containing the B7-H4 transgene or the empty vector (later on referred to as control). Twenty-four hours later the cells were selected with 400 μg/mL G418 and single cell clones were obtained by serial dilutions in 96-well plates. The transfectants were kept under selective pressure throughout their cell-culture period, except for the coculture with T cells. The stability of transgene expression was randomly controlled.

To generate CD8$^+$ primed T cell lines from healthy donors, melanoma cells were transfected with a B7-2–containing plasmid (32), similar as described above.

Stable B7-H3–specific shRNA (cgtgtgctggagaaagatcaa) or irrelevant shRNA (referred to as sh control) expression vectors with a neomycin resistance were transfected into melanoma cells using Lipofectamine 2000 (Invitrogen) and 3 μg of the respective plasmid. Transfected cells were

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treated with 400 μg/mL G418 and in addition, cells were sorted on a FACSvantage (Becton Dickinson) for the lowest B7-H3 expression before using the cells for experiments. The B7-H3 repression on shRNA treatment and sorting was stable for at least 5 months.

T-cell culture

The MART-1–specific T cell clone (A42) was kindly provided by Rivoltini [originally described by Kawakami and colleagues (33)] and was kept as well as expanded for experiments with a modified rapid expansion protocol described by Ho and colleagues (34). In brief, culture medium was RPMI 1640 supplemented with 10% heat inactivated male AB serum (c-c pro), 1% P/S, 1% glutamine, and 1% HEPES. T cells (1×10^5) were incubated with 5×10^6 irradiated (90 Gy) EBV B cells + 2.5×10^7 irradiated (30 Gy) PBMC + 100 U/mL IL-2 and 30 ng/mL αCD3 for 12 days. EBV B cells and peripheral blood mononuclear cells (PBMC) were loaded with 40 μg/mL MART-1 peptide 2 hours before addition of T cells. On day 4 and day 8, the complete or half of the medium, respectively, was exchanged and IL-2 (100 U/mL) was added.

$CD8^+$ primed T cells (T-cell line) were obtained by the following protocol: PBMC were obtained by a Ficoll gradient of buffy coats from healthy donors. Subsequently, $CD8^+$ T cells were isolated by a positive sort with αCD8 Miltenyi beads following the companies' instructions. CD8 T cells (1×10^6) were incubated with 1×10^6 irradiated CD8 depleted PBMC (30 Gy, same donor) and 1×10^5 irradiated B7-2 transfected BUF1088 (90 Gy) in 24-well culture dishes. After 3 days, IL-2 (100 U/mL) was added to the culture. On day 7, $CD8^+$ T cells were restimulated with the same cells and same ratio like the primary culture and IL-2 (100 U/mL) was added on day 3.

Chromium release assay

Cytotoxicity assays were carried out in MART-1 T-cell–specific culture medium using 1×10^6 target cells labeled with 100 μCi^{51} sodium chromat (Hartmann Analytics) for 1 hour at 37°C, 5% CO_2 and washed 3 times. For experiments with MART-1–specific T cells, the transfected tumor cells were pulsed with MART-1 peptide (20 μg/mL) for 1 hour. For blocking experiments, melanoma transfectants were preincubated for 30 minutes at 37°C, 5% CO_2 with 200 μg/mL αHLA class I–specific mAb w6/32 or the corresponding antiimmunoglobulin isotype control. Chrome-labeled cells (2×10^3) were incubated with the respective effectors at different E:T ratios for 4 hours at 37°C. Effector T cells were taken from day 12 (MART-1) or day 21 (primed CD8) cultures or thawed at the day of the assay. Specific lysis was calculated as [cpm (effector cells)–cpm (spontaneous)/cpm (Triton X-100)–cpm (spontaneous)] × 100%.

Cytokine detection by IFN-γ secretion assay and ELISA

Effector T cells were taken from day 12 (MART-1) or day 21 (primed CD8) cultures or thawed at the day of the assay. For experiments with MART-1–specific T cells, the transfected tumor cells were pulsed with MART-1 peptide (20 μg/mL) for 1 hour. T-cell–tumor cocultures were carried out in an E:T ratio of 1:1 for 16 hours at 37°C, 5% CO_2. PMA/ionomycin (10 ng/mL and 1 μg/mL) stimulation served as assay control.

The IFN-γ secretion assay was carried out following the manufacturer's instructions (Miltenyi). ELISAs for IL-2 and TNFα were carried out from culture supernatants according to manufacturer's instructions (eBioscience).

Statistics

Students unpaired *t* test was applied for differences in cytokine production. Mann–Whitney *U* test was used for the comparison of the immunohistochemistry (IHC) score in normal skin, primary tumor lesions, and metastases. The Kaplan–Meier method was used to analyze survival data and the log-rank (Mantel–Cox) test was taken to control differences in patients survival. Mann–Whitney *U* test or Fisher's exact test were applied for checking the association of B7-H3 or B7-H4 expression with clinical parameters. All statistical analyses were carried out using prism GraphPad 5.0 and values of $P < 0.05$ were considered significant.

Results

B7-H3 and B7-H4 expression in metastatic melanoma

IHC was carried out using tissue samples from 29 melanoma patients with complete material of normal skin, primary tumor, and metastases. The obtained sections of melanoma tissues were first analyzed for melanoma marker expression to ensure subsequent analyses of tumor cells in the serial sections. Individual sections from all 29 patients were positively stained (~80% of the tumor cells) with antibodies directed against melanoma marker HMB45 (gp100) and Melan-A (MART-1; Fig. 1A and I, and data not shown; ref. 35), confirming the affiliation to tumor tissue. Stainings with αB7-H3 and αB7-H4 mAb exhibited a high frequency (B7-H3: primary, 100% 29 of 29 and metastases, 97% 28 of 29; B7-H4: primary, 97% 28 of 29 and metastases, 90% 26 of 29) of intracellular and surface expression on tumor cells *in situ* as representatively shown in Figure 1B, C, J, and K and Supplementary Figure S1. Normal skin surrounding the benign lesions of the same patients revealed either no B7-H3 or B7-H4 staining or a very weak signal with a mean IHC score of 1.44 for B7-H3 and 1.21 for B7-H4 (Fig. 2A and B), for all normal skin tissues analyzed. In contrast, the mean IHC score for B7-H3 and B7-H4 of all primary melanoma or metastases analyzed was above 3 and thereby significantly increased when compared with normal skin (Fig. 2A and B). Furthermore, the mean IHC score for both molecules did not differ in the primary tumor versus metastases (Fig. 2A and B). The specificity of the B7-H3 and B7-H4 staining was controlled as described in Materials and Methods and are depicted in Figure 1D, E, and L. According to the quality and quantity of the staining (Materials and Methods), groups of B7-H3 or B7-H4 low (scores 0–3) and high (scores 4 and 5)

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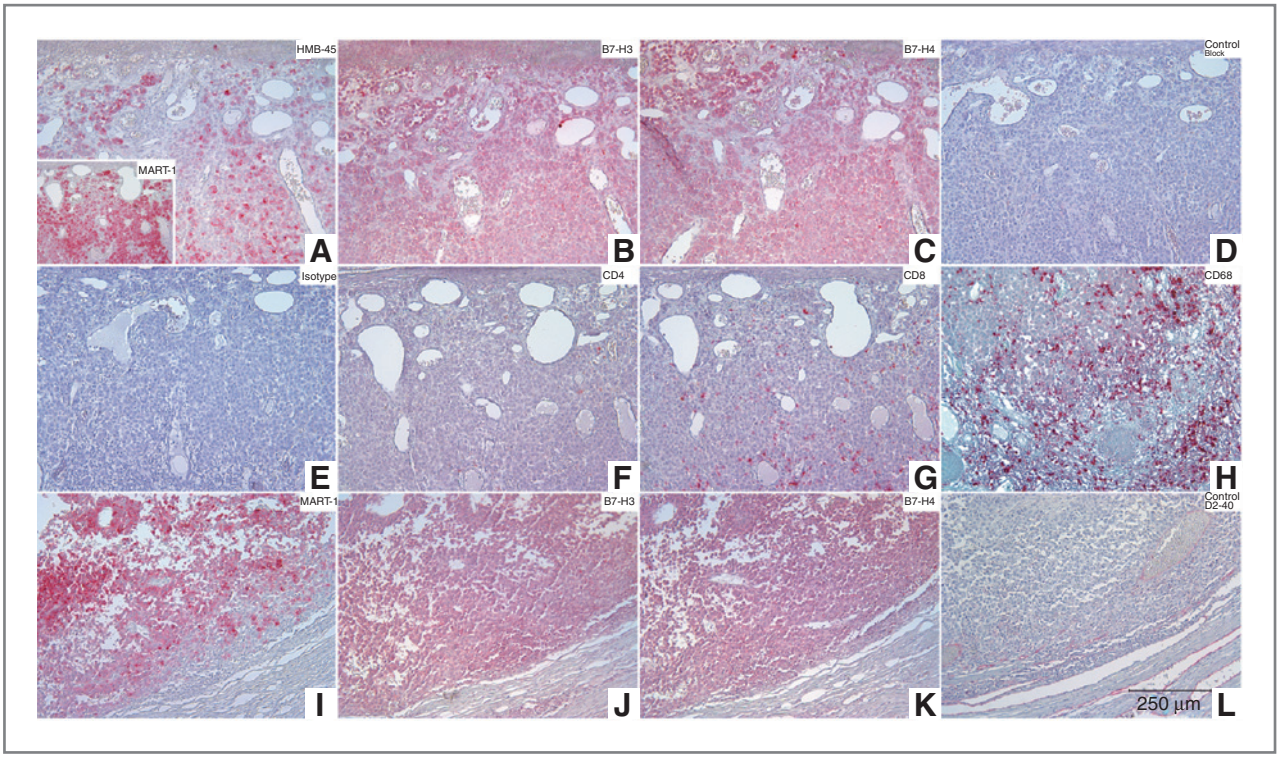

Figure 1. Representative immunohistochemical analysis of B7-H3 and B7-H4 expression in tissue sections of melanoma patients. A–H, primary melanomas: HMB-45, inset MART-1 (A), B7-H3 (B), B7-H4 (C), block control (D), isotype (E), CD4 (F), CD8 (G), and CD68 (H). I–L, melanoma metastases: HMB45 (I), B7-H3 (J), B7-H4 (K), control (D2-40 lymphatic endothelium staining; L). IHC was carried out as described in Materials and Methods.

expression in tumor lesions were identified, respectively. With the exception of 1 tumor lesion, all the other tumor lesions concomitantly expressed B7-H3 and B7-H4 in the primary tumor.

Analysis of immune cell infiltrates in B7-H3 or B7-H4 low and high expressing groups

Taylor and colleagues showed the association of immune cell infiltrates with the clinical outcome of melanoma patients (2). Furthermore, the B7-H3 and B7-H4 expression identified on melanoma cells could directly modulate T cell or NK cell responses, making it important to analyze the immune infiltrate into this tumor. The melanoma lesions analyzed in this study had only marginal T helper cell ($CD4^+$) as well as NK cell ($CD56^+$) infiltration (Fig. 1F and data not shown). The immune cell infiltrate comprised mainly of $CD8^+$ cytotoxic T cells and $CD68^+$ macrophages (Fig. 1G and H). There exists no correlation of B7-H3 expression levels and the frequency of $CD8^+$ or $CD68^+$ immune cell infiltration in primary tumor tissues and metastases (Supplementary Table SI). In contrast, a significant increase in $CD68^+$ immune cell infiltration was detected in the B7-H4 high expressing melanoma (mean of 25.3% $CD68^+$ cells) versus 12.8% $CD68^+$ cells in the B7-H4 low expressing primary tumors (Table 1). $CD8^+$ T-cell infiltration was independent of the B7-H4 expression level in primary tumor as well as metastatic lesions (Table 1).

Lower B7-H4 expression in patients is associated with better survival

With respect to sex, age, Breslow score, and melanoma subtype, no association was found between the level of B7-H3 or B7-H4 expression and the disease course (Table 1 and Supplementary Table SI). B7-H3 expression in melanoma lesions was not associated with patients' survival (Supplementary Table SI). More interesting, even though the number of patients is small, the overall survival of patients with B7-H4 low lesions ($n = 8$) according to analysis on the primary tumor tissue with a median survival of 106.9 month was significantly ($P < 0.05$) higher than that for patients with B7-H4 high lesions ($n = 21$) with a median survival of 42.2 month (Fig. 2C). Of note, at the time of the primary tumor resection, all patients were classified as stage I or II of melanoma. All the 29 patients in our study eventually progressed to stage III or stage IV melanoma, but display a different overall survival. These results suggest a role of B7-H4 expression in the progression of metastatic melanoma.

B7-H3 and B7-H4 expression on melanoma cell lines

To confirm the data obtained with the patients material and to find insights into how B7-H4 might influence patients' survival, a total of 23 melanoma cell lines were analyzed *in vitro* for their expression of B7-H3 and B7-H4. Both molecules were expressed on the mRNA level

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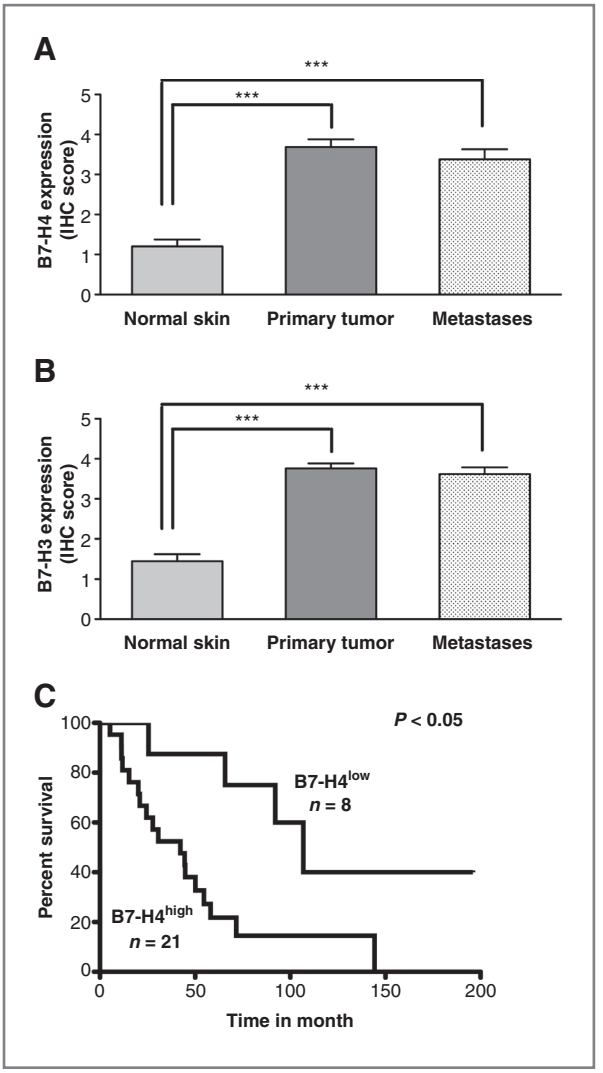

Figure 2. Summarized B7-H3 and B7-H4 expression levels on tissue sections of melanoma patients and correlation of patients' overall survival to B7-H4. A and B, tissue sections of normal skin, primary tumor, and metastases of 29 melanoma patients were analyzed for B7-H3, $P = 0.0001$ (A) and B7-H4, $P < 0.0001$ (B) by immunohistochemistry. C, patients were grouped into low (scores 0–3, $n = 8$) and high (scores 4 and 5, $n = 21$) B7-H4 expression according to immunohistochemistry data in primary tumor sections; $P < 0.05$.

(Supplementary Table SII) although B7-H3 transcript levels exceed those of B7-H4 (data not shown). Concerning B7-H3 protein expression flow cytometry revealed a profound expression of the molecule on the surface of the melanoma cell lines (Fig. 3A). Tumor cell lines of distinct histology with known B7-H3 expression like RCC and ovarian adenocarcinoma (12) served as controls and also stained positive for B7-H3 (Fig. 3A). The specificity of the B7-H3 mAb was additionally confirmed by blockade with the unconjugated form prior to staining with the direct conjugate (Fig. 3C). In contrast, despite B7-H4 mRNA expression (Supplementary Table SII) none of the 23 cell lines analyzed as representatively shown for 2 randomly selected cell lines, expressed B7-H4 on the cell surface (Fig. 3B). Cells of different tumor histology, with known B7-H4 expression like breast carcinoma cells and RCC (17) cells served as controls and showed positive B7-H4 stainings (Fig. 3B). A low, but repeatable staining for B7-H4 on the cell surface was also detectable for EBV-transformed B cells (Fig. 3B; ref. 36). To proof whether melanoma cells express B7-H4 protein at all, intracellular flow cytometry stainings were carried out. The analyses using 2 commercial available monoclonal αB7-H4 antibodies revealed B7-H4 positivity of all cell lines tested (Fig. 3B; Supplementary Table SII and data not shown). The specificity of the αB7-H4 mAb clone MIH43 and clone H74 was controlled via blockade of the staining on the B7-H4 overexpressing BUF1088 cells with the unconjugated form of the same antibody (Fig. 3C and data not shown). In line with data from Kryzek and colleagues in ovarian cancer, IL-4, IL-6, and IL-10 did not induce/increase B7-H4 surface expression on melanoma cells (ref. 37, data not shown). In addition, IFNγ and TNFα also did not modulate B7-H4 expression on melanoma cell lines.

Role of B7-H3 in immune response against melanoma

To define the role of B7-H3 in the antitumor immune response, B7-H3 expression was inhibited to more than 90% on melanoma BUF1088 cells using stable shRNA expression vectors (Fig. 4A). Silencing of B7-H3 did neither influence HLA class I expression nor ICAM-I as shown by their comparable expression on both sh B7-H3- and sh control–treated BUF1088 cells (Fig. 4A). This is important to note, as HLA class I and ICAM-I have an impact on the outcome of antitumor T-cell responses (38, 39). Chromium release assays showed that B7-H3 does not influence TAA-specific $CD8^+$ T cell mediated killing, as the percentage of killing of sh B7-H3- and sh control–treated BUF1088 is comparable (Fig. 4B). The specificity of the MART-1 T cell clone was proven using MART-1_{27-35} and tyrosinase peptide loaded T2 cells (Supplementary Fig. S2). In addition, the IFNγ secretion by MART-1–specific T cells on T-cell–tumor coculture is unaffected by the level of B7-H3 expression (Fig. 4C).

It has been proposed that B7-H3 can protect glioblastoma cells from NK-cell–mediated killing (40). Using preactivated NK cells from healthy donors, no difference in killing of differential B7-H3–expressing melanoma cells was found (data not shown).

B7-H4 on melanoma cell lines does not change $CD8^+$ T-cell–mediated cytotoxicity, but inhibits T-cell–cytokine production

Although the number of $CD8^+$ T cell infiltrates did not differ in the B7-H4 low versus high expressing melanoma cells, still a functional impairment of this cells is feasible, as the activity of tumor infiltrating lymphocytes in the tumor mass is often blocked (23). It has been shown that B7-H4 can inhibit different T-cell functions (12). To define the role for B7-H4 in the T-cell antitumor immune response, B7-H4 was overexpressed in the melanoma cell line

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Table 1. B7-H4 expression and its correlation to clinical parameters in metastatic melanoma patients

	Primary tumor			Metastasis		
	Low, *n* = 8	**High, *n* = 21**	***P***	**Low, *n* = 9**	**High, *n* = 20**	***P***
Sex: F/M	5/3	8/13	0.406	5/4	8/12	0.688
Breslow index	3.25 ± 1.77	4.32 ± 2.31	0.339	3.00 ± 2.01	4.47 ± 2.19	0.088
Subtype	1	4		0	5	
UCM	1	1		1	1	
ALM	5	12		7	10	
SSM	1	4		1	4	
NM						
Age	57.2 ± 19.1	64 ± 15.1	0.527	56.7 ± 14.8	62.9 ± 15.0	0.311
CD8 infiltrates	2.13 ± 0.83	2.05 ± 1.12	0.96	1.56 ± 0.73	1.75 ± 0.55	0.449
CD68 infiltrates	12.8 ± 8.7	25.3 ± 10.3	**0.007**	19.7 ± 9.72	24.3 ± 12.2	0.407

BUF1088, which did not change the expression levels of HLA class I surface antigens nor ICAM-I (Fig. 5A). The costimulatory members of the B7-H family often do not influence the initial priming of T cells, but rather the effector phase (12). Therefore, 2 different effector $CD8^+$ T cells were used for these analyses, the MART-1–specific T cell clone and *in vitro* primed $CD8^+$ T cells. The latter T cells were raised on melanoma cell lines and probably display a broad specificity against melanoma associated antigens. Prior to coculture assays, the surface marker composition was determined by flow cytometry. $CD8^+$ primed T cells analyzed on day 7 after the 2nd restimulation (day 21) suggesting a central memory phenotype of cells ($CD45RA^-$, $CCR7^+$, $CD28^+$, $CD27^+$; ref. 41). In contrast, the MART-1–specific T cells display marker of terminal differentiated effector memory cells ($CD45RA^-$, $CD28^-$, $CD27^-$), but still express the central memory marker CCR7 (Supplementary Fig. S3A). On coculture assays with these $CD8^+$ effector cells, no difference in $CD8^+$ T-cell–mediated killing of B7-H4 overexpressing melanoma cells was detected (Fig. 5B, Supplementary Fig. S3B). In the next step, it was analyzed whether tumor-associated B7-H4 had an influence on T cell cytokine production. A marked inhibition of 40% and 42% of IFNγ production of these

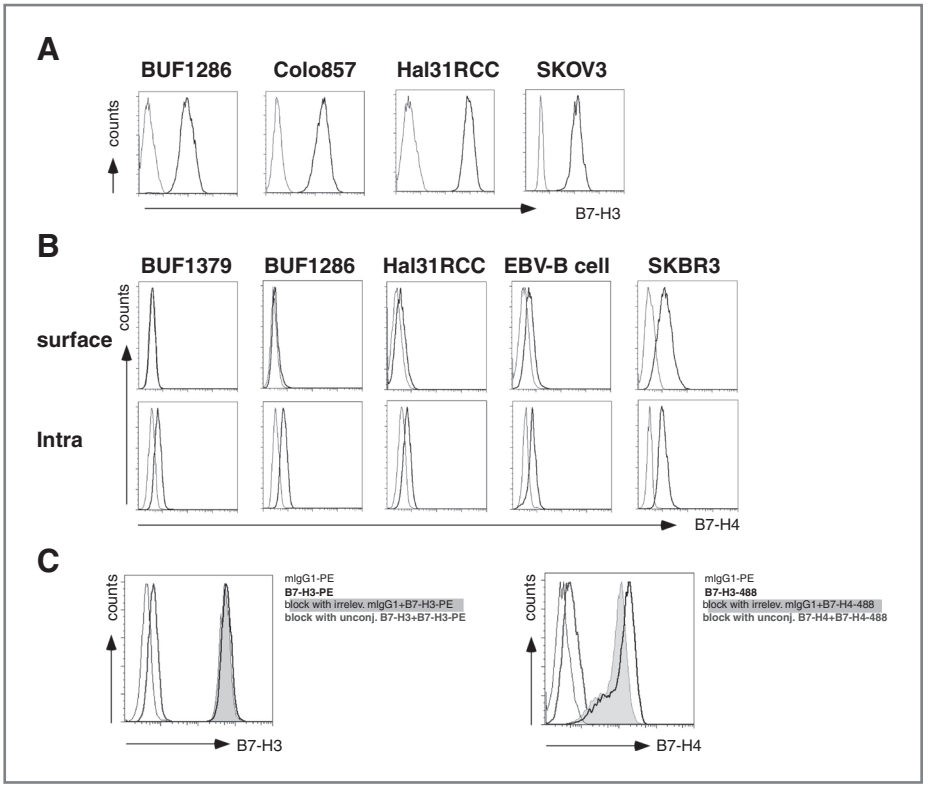

Figure 3. Constitutive B7-H3 and B7-H4 expression on melanoma cell lines. A, surface B7-H3 (bold line) expression on representative melanoma (BUF1286 and Colo857) cell lines and cells of different histology are depicted. Thin line represents a respective isotype control. B, surface and intracellular B7-H4 (bold line, clone MIH43) expression on representative melanoma (BUF1379 and BUF1286) cell lines and on cells of different histology are depicted. Thin line represents a respective isotype control. C, specificity control of B7-H3 and B7-H4 surface staining. These analyses were carried out using melanoma cell lines BUF1383 (B7-H3) and BUF1088 transfected with B7-H4 construct (B7-H4). Representative histograms from at least 3 independent stainings are shown.

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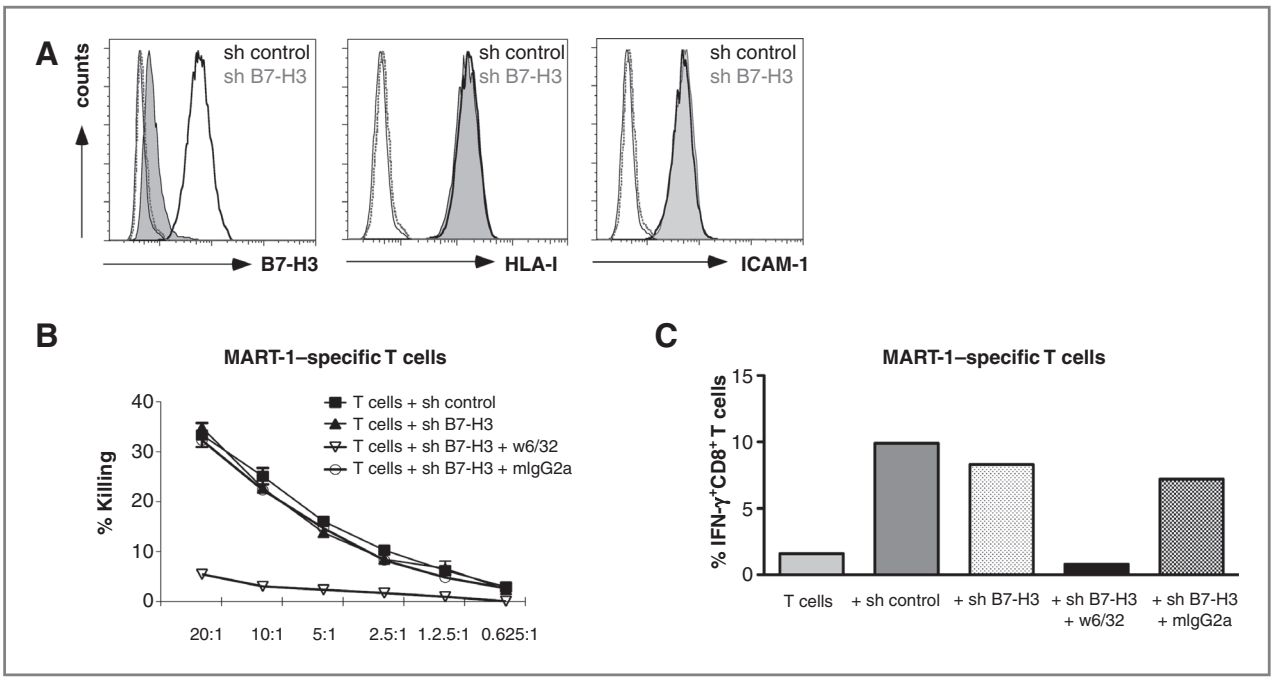

Figure 4. B7-H3 on melanoma cells does not influence TAA-specific CD8 T cell responses. A, transfectants with stable B7-H3 sh plasmids as compared with stable control sh plasmids are shown: black bold line, sh control BUF 1088 with respective staining; black thin line, sh control BUF 1088 isotype control; gray filled curve, sh B7-H3 BUF 1088 with respective staining; gray dotted line, sh B7-H3 BUF 1088 isotype control. Representative histograms from at least 3 independent stainings are shown. B, standard ^{51}Chrom assays for killing of MART peptide loaded BUF 1088 sh B7-H3 transfectant (▲) as compared with sh control transfectant BUF 1088 (■) on coculture with different ratios of MART-1–specific T cells are depicted. MART-1–specific tumor kill is controlled by αHLA-I antibody (w6/32; ▽) and with a respective isotype (mIgG2a) antibody (⊖). Representative data from 3 to 5 independent experiments are shown. C, IFN-γ–specific cytokine secretion assay on coculture of BUF 1088 sh B7-H3 transfectant and sh control transfectant BUF1088 with MART-1–specific T cells was carried out. Results are depicted as percent $CD8^+IFN\gamma^+$ T cells. T-cell–specific cytokine production is controlled by αHLA-I antibody (w6/32) and with a respective isotype (mIgG2a) antibody. Representative data of 1 of 5 similar experiments are shown.

different T cells on coculture with B7-H4 overexpressing melanoma cells, was detected (Fig. 5C). In addition, IL-2 and TNF-α secretion by MART-1–specific T cells was significantly reduced ($P < 0.01$) on coculture with B7-H4 overexpressing melanoma cells (Fig. 5D).

Discussion

The expression and functional relevance of members of the B7 costimulatory family in tumor lesions of distinct histology have been frequently reported (13, 17). Limited data are available for melanoma and in particular, the expression and significance of B7-H3 and B7-H4 molecules have not yet been analyzed in detail.

In this study, immunohistochemistry showed B7-H3 and B7-H4 stainings in primary tumor lesions and metastases. Moreover, there exist a correlation between the level of B7-H4 expression and clinical data, like macrophage infiltration and patient's survival. *In vitro* data using T-cell–tumor coculture revealed an inhibitory effect for tumor B7-H4 on cytokine production of different $CD8^+$ T effector cells.

B7-H4 expression was detected with a high frequency ranging from 97% 28 of 29 in the primary tumors to 90% 26 of 29 in the metastases (Fig 1C and K and Table 1). Theses data were in accordance to the high frequency of B7-H4 in breast carcinoma lesions with a positive staining pattern of 95% in the primary and 98% of metastatic lesions (42), other studies analyzing RCC (43) and lung cancer (44) show B7-H4 expression in only 60% and 43% of the cases, respectively. In contrast to our data the groups of Choi and Gajewski did not detect B7-H4–positive melanoma lesions (13, 45). This discrepancy might be explained by the cohort of melanoma patients analyzed, which was not described in both reports as well as by the methodology used. Chen and colleagues employed frozen sections for IHC, whereas our study was conducted on paraffin-embedded tissues. In addition, a different B7-H4–specific mAb and a distinct protocol were used.

Comparable to B7-H4, B7-H3 expression was detected in melanoma lesions with a positive B7-H3 staining pattern for 100% of the primary and 97% of the metastatic lesions (Table I and Supplementary Table SII). In other tumor entities, the frequency of tumor B7-H3–positive cases varied from 46% in RCC (22) to 96% in prostate cancer (46). As described by others (42, 47), a low expression of both B7-H3 and B7-H4 with a score of 1 to 2 was detected in normal skin surrounding the benign lesions of the same patients (Fig. 2A and B).

In accordance with findings in gastric (48) and RCC (43) patients, an increased survival of melanoma patients with

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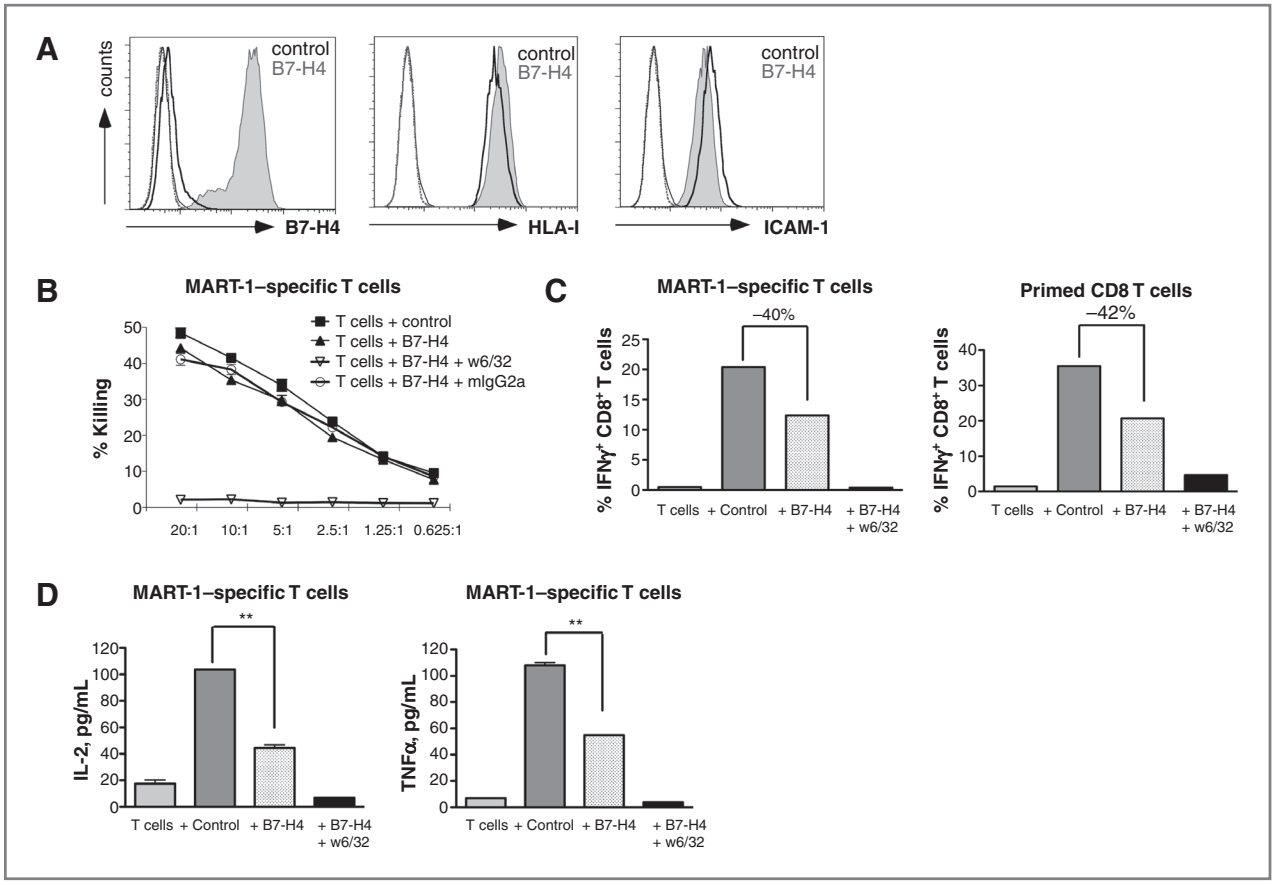

Figure 5. B7-H4 expression on melanoma cells does not influence TAA-specific CD8 T cell kill, but inhibits cytokine production. A, overexpression variant for B7-H4 as compared with empty vector (control) is shown: black bold line, control-transfected BUF 1088 with respective staining; black thin line, control-transfected BUF 1088 isotype control; gray filled curve, B7-H4–transfected BUF 1088 with respective staining; gray dotted line, B7-H4 transfected BUF 1088 isotype control. Representative histograms from at least 3 independent stainings are shown. B, standard ^{51}Chrom release assays for killing of MART peptide loaded BUF 1088 B7-H4 transfectant (▲) as compared with control transfectant (■) on coculture with different ratios of MART-1–specific T cells are depicted. MART-1–specific tumor kill is controlled by αHLA class I antibody (w6/32; ▽) preincubation and with a respective isotype (mIgG2a) antibody (⊖). Representative data from 3 to 5 independent experiments are shown. C, IFN-γ–specific cytokine secretion assay on coculture of BUF 1088 B7-H4 transfectant and control transfectant with MART-1 T cells or primed CD8 T cells was carried out. Results are depicted as percent $CD8^+IFN\gamma^+$ T cells. T-cell–specific cytokine production is controlled by αHLA-I antibody (w6/32) preincubation. Representative data of 1 of 4 (MART-1 T cells) and 1 of 3 (CD8 primed) similar experiments are shown. D, IL-2 and TNF-α production by MART-1–specific T cells on coculture with BUF 1088 B7-H4 transfectants and control transfectants of BUF 1088 cells were analyzed by standard ELISAs. MART-1–specific cytokine production is controlled by αHLA-I antibody (w6/32) preincubation. Representative data of 1 of 3 similar experiments are shown.

lower B7-H4 expressing tumor was found (Fig. 2C). It is noteworthy that the number of cases analyzed in melanoma is rather low, which is due to the selection criteria (material of normal skin, primary tissue, and metastases of the same patients) and needs to be extended in the future by focusing on primary melanoma tissues. In line with the IHC in melanoma lesions, B7-H4 mRNA and protein expression were found on melanoma cell lines (Supplementary Table SII and Fig. 3). Noteworthy, B7-H4 surface expression was not detectable on the melanoma cell lines, but on ovarian cells, whereas intracellular B7-H4 was found in all the melanoma cell lines tested (Fig. 3 and Supplementary Table SII). The mechanism retaining B7-H4 in the cytosol has not yet been investigated.

To understand the underlying immune mechanism, the frequency and composition of the tumor immune infiltrates of the melanoma cases in our cohort were determined. Predominantly $CD8^+$ and $CD68^+$ cells were detected in the lesions, but only $CD68^+$ and not $CD8^+$ infiltrates were positively associated with higher B7-H4 expression (Table 1). The number of lymphocyte infiltrates has been correlated to patient's survival in some studies (2, 49), but not in others (50, 51). Even more important than the frequencies of immune infiltrates is the functionality of the immune cells (52). Due to the observed inhibitory effect of tumor B7-H4 on T cell function *in vitro* (Fig. 5C and D), we suggest that B7-H4 expression in tumor lesions might also modulate $CD8^+$ T cell function *in vivo*.

Overexpression of B7-H4 on melanoma cells resulted in an inhibition of T-cell functions (Fig. 5C and D), which was comparable to the inhibitory role of B7-H4 in other settings (13). In contrast to Ou and colleagues (53), our data

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revealed that B7-H4 had no direct influence on the killing ability of $CD8^+$ T cells, but on cytokine production, in particular of IFN-γ, TNF-α, and IL-2 (Fig. 5B–D). Interestingly, also murine tumor B7-H2 had no direct influence on killing of mastocytoma cells, but still enhanced tumor rejection (54). In addition, CD28 costimulation does not affect lysis of TCR-engrafted T cells, but did have an impact on cytokine responses (55). Inflammatory cytokines have a number of diverse functions in the antitumor immune response (56). They not only serve as growth factors, but IFN-γ and TNF-α exhibit chemotactic potential via direct (at least TNF-α) or indirect mechanisms, the latter mediated by IL-8 and MCP-1 enhancement (57, 58). Inhibition of monocytes/macrophage recruitment to the tumor site appeared not to be the defect in the lesions described in this study, as high B7-H4 expression on tumors was associated with increased macrophage density (Table 1). Therefore, other mechanisms might lead to the reduced survival in the high B7-H4 expressing melanoma cases. Possibly, reduced IFN-γ levels at the tumor site might impact the tumor escape by lower tumor HLA class I and adhesion molecule expression, which in turn might leads to an even more decreased T-cell response (38).

Furthermore, other studies reported a suppressive effect of $B7\text{-}H4^+$ tumor–associated macrophages on DC-induced TAA-specific $CD8^+$ T cell responses in triple cultures of ovarian cancer (37). Due to the lack of surface B7-H4 expression in ovarian cancer lesions, the inhibitory role of B7-H4 in ovarian cancer might be due to B7-H4 expression on TAMs (37). In contrast, other groups detected B7-H4 expression on the cell surface of ovarian cancer lesions as well as on cell lines *in vitro* (59, 60) suggesting the possibility of a direct interaction between $CD8^+$ T cells and B7-H4 on tumor cells also in ovarian cancer patients. A B7-H4–positive immune cell infiltrate was detected in melanoma lesions, which does not rule out the possibility that tumor-associated B7-H4–positive macrophages might also confer suppressive activity on tumor infiltrating $CD8^+$ T cells in melanoma (data not shown). Because the B7-H4–positive immune infiltrate was not further characterized, the $CD8^+$ T cells themselves might express B7-H4, which is in line with published data (61).

Despite no association between clinical parameters and immune cell infiltration with B7-H3 expression levels was found in melanoma patients (Supplementary Table SI), a role for B7-H3 in melanoma could not be excluded, in particular, as B7-H3 expression on tumor cells could act on receptor bearing nonimmune cells like fibroblasts. The recently identified receptor TLT-2 exhibits a broad expression on cells (12). In addition, even though B7-H3 was also expressed on tumor cells of ovarian cancer, only the B7-H3 expression on the tumor vasculature was associated with patients' survival (62). B7-H3 on tumor cells did enhance $CD8^+$ T cell effector functions in humans (63) *in vitro*. These findings are in accordance with patient data documenting a positive correlation of B7-H3 with increased survival in gastric and pancreatic tumor patients (21, 47). In contrast, an inhibitory role for B7-H3 on T cell effector functions has been documented, but the assays were not carried out with tumor cells, instead with APCs or with B7-H3Ig (16, 64). This B7-H3–induced inhibitory functions would be in line with a positive association of increased mortality in RCC patients (22). Certainly a distinct receptor expression in the different experimental settings and more important in the different tumor entities might be responsible for these discrepancies. B7-H3 protein expression on a small number of melanoma cell lines had been previously shown *in vitro* (65). In our study, all tumor cell lines tested were unambiguously B7-H3 mRNA and surface protein positive (Supplementary Table SII and Fig. 3). Human TAA–specific T-cell–tumor coculture assays with modulated B7-H3 revealed no differences in T-cell responses (Fig. 4).

In summary, an association of B7-H4 expression with increased death in melanoma patients was found. The *in vitro* data suggest as underlying mechanism a decreased cytokine production of TAA-specific T cells on strong B7-H4 expression on tumor cells. This could subsequently lead to a reduced T-cell proliferation and lower HLA class I surface expression at the tumor site that finally might result in tumor immune evasion. These data presented here could have an important impact on the consideration of new therapeutic strategies against metastatic melanoma.

Disclosure of Potential Conflicts of Interest

There is no conflict of interest with any of the authors concerning commercial affiliations, consultancies, stock or equity interests, and patent-licensing arrangements.

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Clinical Cancer Research

B7-H4 Expression in Human Melanoma: Its Association with Patients' Survival and Antitumor Immune Response

Dagmar Quandt, Eckhard Fiedler, Diana Boettcher, et al.

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Appendix

Selbstständigkeitserklärung

Hiermit erkläre ich an Eides statt, dass ich die hier vorliegende Habilitationsschrift selbständig und nur unter Verwendung der angegebenen Literatur und Hilfsmittel angefertigt habe.
Ein Habilitationsverfahren wurde an keiner anderen Universität eröffnet oder beantragt.
Frühere Habilitationsversuche sind nicht unternommen worden.

Halle, den 12.04.2018 Dr. Dagmar Quandt

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